EFFECT OF INSULIN-LIKE GROWTH FACTOR-1 ON DEVELOPMENT AND POST-TRANSFER SURVIVAL OF BOVINE EMBRYOS PRODUCED IN VITRO

By

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To my parents and family.

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bST	Bovine somatotropin
CIDR	Controlled internal drug release device
COC	Cumulus-oocyte complex
Dc	Desmocollin
DIM	Days in milk
DNA	Deoxyribonucelic acid
DPBS	Dulbecco's phosphate buffered saline
Ecad	E-cadherin
FSH	Follicle stimulating hormone
GnRH	Gonadotropin-releasing hormone
GLUT	Glucose transporter
hCG	Human chorionic gonadotropin
Hsp	Heat shock protein
IETS	International embryo transfer society
IFN	Interferon
ICM	Inner cell mass
IGF-1	Insulin-like growth factor-2
IGF-1R	Insulin-like growth factor receptor
IGF-2	Insulin-like growth factor-2
IGF-2R	Insulin-like growth factor-2 receptor
IGFBP	Insulin-like growth factor binding protein
KSOM	Potassium simplex optimized medium

mRNA	Messenger ribonucleic acid
Na/K	Sodium/potassium ATPase
OCM	Oocyte collection medium
OMM	Oocyte maturation medium
RT	Reverse transcription
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGF	Prostaglandin $F_{2\alpha}$
Plako	Plakophilin
PVP	Polyvinylpyrrolidone
RIA	Radioimmunoassay
sHLA-G	Soluble human leukocyte antigen-G
TALP	Tyrode's albumin lactate pyruvate
TE	Trophectoderm
TCM	Tissue culture medium
TMR	Total mixed ration
TUNEL	Terminal deoxynucleoytidyl transferase mediated dUTP nick end labeling
VEGF	Vascular endothelial growth factor

Abstract of Dissertation Presented to the Graduate School
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In vitro embryo production has great potential as a tool for optimizing genetic selection, improving fertility and enhancing breeding schemes in beef and dairy production systems. Despite its potential, the use of in vitro embryo production is limited by several technical problems, including reduced embryo survival following transfer. One approach for improving survival post-transfer of in vitro produced embryos is to modify culture media with growth factors. Recently, the addition of IGF-1 to bovine embryo culture increased pregnancy and calving rates in heat-stressed, lactating dairy cows. A series of experiments was conducted to determine how IGF-1 promotes the survival of in vitro produced bovine embryos after transfer.

The production of embryos in vitro can alter several aspects of embryo physiology, including gene expression. An experiment was conducted to determine whether addition of IGF-1 to embryo culture could alter the abundance of several developmentally important gene transcripts. Treatment of embryos with IGF-1 increased the relative abundance of transcripts for Na/K, DcII, Bax, and IGFBP3, while decreasing the abundance of Hsp70 and IGF-1R transcripts. In contrast, IGF-1 supplementation had no effect on blastocyst cell number, cell allocation, or the proportion of apoptotic blastomeres.

Two experiments were conducted to determine whether IGF-1 treatment could improve embryo survival around the time of maternal recognition of pregnancy. In the first experiment, embryos were transferred to recipients in groups. There was no effect of IGF-1 on embryo survival at day 14. Moreover, IGF-1 did not affect embryo length, stage, embryonic disc formation or IFN-τ secretion. In the second experiment, each recipient received a single embryo. There was a tendency for IGF-1 to increase embryo survival at day 14. However, as in experiment 1, there was no effect of IGF-1 on embryo length, stage or IFN-τ secretion.

A field trial was conducted to determine whether the effect of IGF-1treatment on embryo survival reported previously was a general effect of IGF-1 or one specific to heat stress. Pregnancy and calving rates were increased for IGF-1 embryo recipients in the summer, but not during the winter. Recipients that received IGF-1 treated embryos in the summer had significantly lower pregnancy loss between day 21 and day 28 of gestation than recipients that received control embryos.

Taken together, these results indicate that IGF-1 can increase pregnancy and calving rates in heat-stressed lactating dairy cows, but such an effect does not occur when recipients are not heat-stressed. The ability of IGF-1 to increase pregnancy rates does not appear to involve an enhanced anti-luteolytic capacity during the period of maternal recognition of pregnancy. On the other hand, IGF-1 actions on blastocyst gene expression may be important for improved embryo survival and could lead to the identification of markers for embryo survival.

PREFACE

The birth of the first calf following in vitro fertilization was reported by Brackett and colleagues in 1982. Since that time, in vitro embryo production has become a widely used assisted reproductive technology in cattle, with several companies around the world offering commercial embryo production services. According to the International Embryo Transfer Society, more than 265,000 in vitro produced bovine embryos were transferred worldwide in 2005 (Thibier, 2006).

The road leading to the current state of in vitro embryo production in cattle began more than 40 years ago when Edwards first reported the in vitro maturation (completion of meiosis I) of bovine oocytes collected from non-ovulatory stage follicles in 1965. In the time since this initial report, much research has been devoted to developing and improving the process of in vitro embryo production. As a consequence, a great deal of knowledge has been accumulated about the regulation of early embryo development in vitro, including the importance of the oocyte (Sirard et al., 2006), the role of media components such as amino acids (Thompson, 2000), and the effect of oxygen tension (Harvey, 2006), among others.

Although much is known about the regulation of embryo development in vitro, very little is know about how the maternal reproductive tract regulates embryo development and survival in vivo. While embryos produced in vitro are exposed to a relatively static media composed of salts, energy substrates and amino acids, embryos derived in vivo are exposed to a complex, constantly changing, milieu of molecules, including hormones, cytokines and growth factors. This dissertation will focus on one of these molecules, insulin-like growth factor-1, and its actions on embryo development in vitro and subsequent survival following transfer.

CHAPTER 1
LITERATURE REVIEW

In Vitro Embryo Production in Cattle

Over the past 25 years, since the birth of the first calf following in vitro fertilization (Brackett et al., 1982), significant advances in the techniques for in vitro embryo production have been made. Such improvements have led to a dramatic increase in the use of in vitro produced embryos in the cattle industry. According to records collected by the International Embryo Transfer Society (IETS), more than 265,000 in vitro produced embryos were transferred worldwide in 2005 compared with less than 42,000 in 2000, a more than six-fold increase in 5 years (Thibier, 2001, 2006). Further increases in the future are likely given the potential applications of in vitro embryo production systems within the beef and dairy industries.

While there is great potential for in vitro embryo production, the actual use of this technology is still very limited. According to the IETS, in 2005, only 30% of all embryos transferred worldwide were produced in vitro (Thibier, 2006). It is well recognized that embryos produced in vitro differ from their in vivo derived counterparts in terms of morphology and physiology. Such differences can affect the post-culture viability of bovine embryos which limits the use of in vitro embryo transfer in commercial settings.

Potential Applications of In Vitro Embryo Technologies

Enhance genetic selection

In vitro embryo technologies have great potential for improving the rate of genetic gain for quantitative traits important for meat and milk production. Both the intensity and accuracy of selection for quantitative traits can be improved through the use of in vitro embryo production (Hansen and Block, 2004). In addition, in vitro embryo production systems can reduce the generation interval through the production of embryos from pregnant animals (Kruip et al., 1994)

and there is also the promise of producing embryos from prepuberal heifers, although problems with oocyte competence need to be addressed (Salamone et al., 2001). Further improvements in genetic gain may also be possible since recent reports indicate that oocytes can be produced from stem cells (Kehler et al., 2005) or derived from cells present in bone marrow (Johnson et al., 2005). Such procedures may eventually allow for an unlimited pool of oocytes from genetically superior females.

There is also potential to optimize genetic selection through the application of in vitro embryo technologies. The use of techniques for preimplantation genetic diagnosis (Bredbacka, 2001; Moore and Thatcher, 2006) can allow for selection of embryos based on their specific allelic inheritance prior to transfer. Several genetic markers have been identified, including markers for milk production traits (Spelman et al., 2002; Freyer et al., 2003), growth and carcass traits (Stone et al., 1999; Casas et al., 2000) and recently, fertility (Garcia at al., 2006). Another genetic trait that is of great importance, particularly in the dairy industry, is sex. While embryo sexing using the polymerase chain reaction has become very common in the commercial embryo transfer industry (Lopes et al., 2001), the advent of sexed semen technology (Seidel, 2003) provides another strategy for skewing sex ratio that has many potential applications in cattle production (Hohenboken, 1999). In vitro embryo production systems provide a more practical approach for the use of sexed semen because more embryos can be produced with sexed semen in vitro than by using superovulation (Bousquet et al., 1999)

Improve fertility

The fertility of lactating dairy cattle has declined over the past 40-50 years (Butler, 1998; Royal et al., 2000; Lucy et al., 2001; Lopez-Gattius, 2003). While the causes of infertility are not fully understood, lactation is associated with reduced oocyte competence (Snijders et al., 2000) and poor early embryo development (Sartori et al., 2002). These problems could

potentially be bypassed through the use of in vitro embryo transfer. The use of oocytes collected from abbatoir-derived ovaries can be used as an inexpensive source of genetic material for producing embryos for large scale embryo transfer breeding schemes. While there are producer concerns about the genetic make-up of abbatoir-derived oocytes, a study by Rutledge (1997) indicates that the genetic merit of cows sent to slaughter is only slightly lower than for the average cow in the herd of origin. Moreover, the ability to produce hundreds of embryos with only a few straws of semen allows for genetic improvement by utilizing semen from genetically valuable sires that in other instances, would be too expensive. To date, few studies have directly compared the pregnancy rates obtained with artificial insemination versus embryo transfer in lactating dairy cattle (Putney et al., 1989; Ambrose et al., 1999; Drost et al., 1999; Al-Katanani et al., 2002). The use of embryo transfer in situations where pregnancy rates to artificial insemination are above average does not seem to provide any increase in fertility (Sartori et al., 2006). In contrast, however, in cases where pregnancy rates to artificial insemination are low, such as during heat stress, in vitro embryo transfer can be effective in improving fertility in lactating dairy cows (Ambrose et al., 1999; Al-Katanani et al., 2002).

Optimize Breeding Schemes

Production of embryos in vitro also has potential for enhancing crossbreeding schemes. The use of crossbreeding to take advantage of heterosis is commonly used in beef production systems. While seldom used for dairy cattle production in the United States, crossbreeding has received renewed attention recently (McAllister, 2002; Heins et al., 2006a; Heins et al., 2006b). Production of F_1 crossbred embryos in vitro for transfer to F_1 recipients could improve crossbreeding schemes by eliminating the loss of heterosis and increased phenotypic variation that occurs when F_1 females are mated to purebred or crossbred sires (Rutledge, 2004).

More than 50% of the costs associated with beef production are derived from maintenance of the mother cow in single calving herds (Dickerson, 1970, 1978). The use of embryo transfer to induce twinning in beef cattle could be important for increasing the efficiency of beef production (Guerra-Martinez et al., 1990). Although induced twinning is not routinely used in beef cattle production systems, the precipitous decline in land for agriculture use and continued population growth may necessitate the use of such schemes in the future. As with producing embryos in vitro to mitigate problems of infertility in dairy cattle, the production of embryos in vitro for induced twinning represents a more practical alternative compared to superovulation. There is also potential for in vitro embryo production and induced twinning in dairy production systems. A recent study incorporated in vitro embryo transfer with sexed semen and induced twinning in beef cattle to produce Holstein heifers as replacements for dairy operations (Wheeler et al., 2006)

Technical Limitations to Use of Embryos Produced in Vitro

Sensitivity to cryopreservation

A major limitation to the widespread use of in vitro embryo production systems in the beef and dairy industries is an inability to efficiently cryopreserve embryos produced in such systems. In vitro produced bovine embryos are more sensitive to cryopreservation than embryos produced in vivo (Pollard and Leibo, 1993; Guyader-Joly et al., 1999; Enright et al., 2000; Rizos et al., 2003). In addition, pregnancy rates obtained with frozen-thawed in vitro produced embryos are consistently lower than for embryos produced by superovulation (Hasler et al., 1995; Agca et al., 1998; Ambrose et al., 1999; Al-Katanani et al., 2002).

Post-transfer development and survival

The transfer of bovine embryos produced in vitro is associated with an increased frequency of abnormalities related to embryonic, fetal, placental and neonatal development. These developmental errors include a wide range of phenotypes including increased rates of embryonic mortality and abortions, production of large fetuses and calves, alterations in development of the allantois, a sex ratio skewed toward males; increased proportion of calves with congenital malformations, and increased neonatal abnormalities (Farin et al., 2001; Farin et al., 2006). These abnormalities had been collectively termed large offspring syndrome, but recently have been more appropriately renamed abnormal offspring syndrome (Farin et al., 2006).

Regardless of whether embryos have been cryopreserved or not, bovine embryos produced in vitro are associated with reduced embryo survival rates following transfer. Pregnancy rates following the transfer of in vitro produced bovine embryos are reduced compared to those obtained following the transfer of in vivo derived embryos (Hasler et al., 1995; Farin and Farin, 1995; Drost et al., 1999).

In addition to problems with reduced pregnancy rates, embryos that survive to the fetal period are more likely to be lost. While pregnancy loss after the first two months of gestation for superovulated embryos is generally less than 5% (King et al., 1985; Hasler et al., 1987), pregnancy loss after day 40 of gestation for in vitro produced embryos has ranged from 12% to 24% (Agca et al., 1998; Hasler, 2000; Block et al., 2003). In two studies in which abortion rate was compared between cows that received an embryo derived in vivo or an in vitro produced embryo, abortion rates were increased for cows that received bovine embryos produced in vitro (Hasler et al., 1995; van Wagtendonk de-Leeuw et al., 2000).

The increased fetal loss that characterizes pregnancies from embryos produced in vitro is most likely related to the abnormal fetal and placental development of in vitro produced bovine

embryos. Farin and coworkers (2006) have done extensive work comparing fetuses and placentae derived from in vitro produced embryos to those derived from in vivo produced embryos. Fetuses produced from in vitro produced embryos are heavier at day 222 of gestation than fetuses derived from in vivo produced embryos (Farin and Farin, 1995; Miles et al., 2004; Crosier et al., 2002). In addition, the fetuses derived from in vitro produced embryos are characterized as having skeletal measurements that are disproportionate to their body weight (Farin and Farin et al., 1995), as well as, altered development of skeletal muscle and reduced abundance of myostatin mRNA (Crosier et al., 2002). Placentae at day 70 of gestation from embryos produced in vitro in modified synthetic oviductal fluid were heavier, had fewer placentomes, and lower placental efficiency (fetal weight/placental weight) than for embryos produced following superovulation (Miles et al., 2005). Placentomes in the in vitro group also had decreased density of blood vessels and also a decreased expression of vascular endothelial growth factor mRNA in cotyledonary tissue. Characterization of placentae during late gestation indicate that at day 222 the proportional volume of blood vessels in the maternal caruncles and the ratio of blood vessel volume density to placentome surface area were increased for in vitro produced embryos (Miles et al., ,2004). These results suggest that, at least in some cases, increased fetal size may be compensated for by an increased vascular blood network in the placentomes. However, the production of embryos in vitro is associated with an increase in hydroallantois (Hasler et al., 1995; van Wagtendonk de-Leeuw et a., 1998, 2000), for which the fetus and placenta cannot effectively compensate (Farin et al., 2006)

Given the abnormalities in fetal and placental development described above, it is not surprising that calves produced from in vitro produced embryos have increased calf birth weights compared to embryos derived in vivo (Behboodi et al., 1995, Jacobsen et al., 2000, van

Wagtendonk de-Leeuw et a., 1998, 2000, Bertolini et al., 2002b). Increased calf birth weights are associated with an increase in dystocia and cesarean sections (Behboodi et al., 1995; Kruip and den Daas, 1997; van Wagtendonk de-Leeuw et a., 1998, 2000) as well as perinatal mortality (Behboodi et al., 1995; Schmidt et al., 1996; van Wagtendonk de-Leeuw et a., 1998, 2000). In addition, calves that are produced from the transfer of in vitro produced embryos are associated with a sex ratio skewed toward males (van Wagtendonk de-Leeuw et a., 1998, 2000), increase in congenital malformations (Schmidt et al., 1996; van Wagtendonk de-Leeuw et a., 1998, 2000), and altered organ development (McEvoy et al., 1998).

Potential causes

It is well recognized that in vitro produced embryos differ markedly from their in vivo derived counterparts in terms of ultrastructure (Crosier et al., 2001; Fair et al.., 2001; Rizos et al., 2002), metabolism (Khurana and Niemann, 2000), and gene expression (Farin et al., 2004; Lonergan et al., 2006). It is likely that many of these differences contribute to the problems described above. At the ultrastructural level, embryos produced in vitro are associated with an increase in cytoplasmic lipid content, alterations in the number and morphological characteristics of mitochondria, and a reduced number of microvilli and intercellular contacts compared to embryos produced in vivo (Crosier et al., 2001; Fair et al., 2001; Rizos et al., 2002). Khurana and Niemann (2000) evaluated the metabolic activity of in vitro produced and in vivo derived embryos and reported that in vitro produced blastocysts produced 2-fold more lactate than blastocysts produced in vivo indicating a major difference in the metabolism of glucose between the two groups of embryos. Several studies have also evaluated the effect of in vitro embryo production of gene expression patterns in bovine blastocysts (Farin et al., 2004; Lonergan et al., 2006). In general, these studies indicate that culture can increase the abundance of heat shock protein 70 (Hsp70; Lazzari et al., 2002; Sagirkaya et al., 2006), increase levels of the pro-

apoptotic protein Bax (Rizos et al., 2002) and decrease the abundance of the tight junction protein connexin-43 (Wrenzycki et al., 1996; Wrenzycki et al., 1998; Rizos et al., 2002). Bertolini and coworkers (2002a) also reported that in vitro produced embryos have increased insulin-like growth factor-2 (IGF-2) and reduced levels of IGF-2R transcript levels. It is important to note that discrepancies between studies with respect to other genes was observed (Bertolini et al., 2002a; Lazzari et al., 2002 Sagirkaya et al., 2006). Moreover, it has been reported that different culture media can have different affects on the abundance of certain genes (Wrenzycki et al., 1999; Yaseen et al., 2001; Lazzari et al., 2002; Rizos et al., 2002; Rizos et al., 2003; Sagirkaya et al., 2006).

It is not clear what affect these differences have on subsequent survival following transfer but it is likely that they contribute to the reduced embryonic or fetal survival of in vitro produced bovine embryos (Hasler et al., 1995; Farin and Farin et al., 1995; Drost et al., 1999). In particular, alterations in embryo function caused by embryo culture may affect early conceptus development around the time of maternal recognition of pregnancy. Bertolini and colleagues (2002a) found that conceptus length at day 16 was decreased for in vitro produced embryos compared to in vivo produced embryos. While Farin and others (2001) reported that conceptus length on day 17 was increased for in vitro produced embryos, they also found that a greater percentage of in vitro produced conceptuses were degenerate. The discrepancies between these studies in terms of conceptus length may be attributed to differences in the survival status of the embryos. For instance, embryos recovered on day 16 most likely represented a population of conceptuses prior to maternal recognition of pregnancy while those that were recovered on day 17 represented a population that had survived luteolysis, thus the shift in conceptus length. In addition, culture condtions have been reported to affect the proportion of day 14 conceptuses that

have a viable embryonic disc (Fischer-Brown et al., 2005). Such an effect could help to explain increases in fetal loss associated with in vitro produced embryos.

While it is clear that the production of embryos in vitro can have long term affects on fetal, placental and neonatal development, the precise reasons for these alterations are not fully understood. One hypothesis is that manipulation of the early embryo during in vitro embryo production alters the expression of imprinted genes. Consistent with this idea, IGF-2 mRNA abundance was altered in day 70 bovine fetuses derived from in vitro produced embryos compared to fetuses that developed from superovulated embryos (Blondin et al., 2000). Moreover, expression of IGF-2 and IGF-2 receptor were also altered in bovine embryos produced in vitro (Bertolini et al., 2002a). Thus, it is possible that bovine embryo production in vitro can affect the methylation patterns that regulate monoallelic expression of imprinted genes. While this has not been reported for in vitro produced bovine embryos, such an effect has been reported in mice (Khosla et al., 2001) and sheep (Young et al., 2001).

The expression of non-imprinted genes can also be altered by in vitro embryo production (Bertolini, 2002a ; Crosier et al., 2002; Miles et al., 2004; Miles et al., 2005). Following fertilization, the paternal DNA undergoes an active, rapid process of demethylation while the maternal DNA undergoes a passive demethylation. During this period, epigenetic marks on non-imprinted genes are erased (Morgan et al., 2005). Embryonic methylation patterns are re-established during development to the blastocyst stage in cattle (Reik et al., 2001; Li, 2002) by the actions of two enzymes, DNA methyltransferase 3a and 3b (Reik et al., 2001; Reik et al., 2003). It is also possible that embryonic manipulation as part of in vitro embryo production could affect the re-methylation of non-imprinted genes and thereby alter post-transfer survival

and development. A recent study indicates that certain bovine embryo culture media can alter the abundance of DNA methyltransferase 3a (Sagirkaya et al., 2006).

As mentioned earlier, it is clear that the differences observed between in vitro produced and in vivo derived blastocysts can affect cryosurvival (Rizos et al., 2002). Changes in gene expression (Rizos et al., 2002, 2003) as well as ultrastrucure (Abe et al.,1999; Fair et al., 2001; Abe et al., 2002) have been associated with a reduced capacity to survive following cryopreservation. In particular the accumulation of lipid droplets in the cytoplasm of embryos produced in vitro appears to reduce cryotolerance. Reduction in lipid content, either by centrifugation (Diez et al., 2001) or by using metabolic inhibitors (De La Torre-Sanchez et al., 2006a, 2006b), can improve survival following cryopreservation.

Alterations in sex ratio caused by in vitro embryo production appear to be related to embryo culture conditions rather than the preferential survival of male embryos after transfer. In vitro, male embryos develop faster than female embryos (Avery et al., 1991; Xu et al., 1992; Gutierrez-Adan et al., 2001). There is some indication that this effect may be the result of glucose in the culture medium (Larson et al, 2001). However, even in medium without glucose, 68% of day 7 expanded blastocysts were male and glucose-free medium did not alter the sex ratio in favor females until day 9-10 after fertilization (Gutierrez et al., 2001). The ability to used sexed semen efficiently for in vitro embryo production as mentioned above offers one strategy to overcome this problem (Wilson et al., 2005; Wheeler et al., 2006; Wilson et al., 2006).

Strategies to Improve Post-Transfer Survival of Bovine Embryos Produced In Vitro

In general, there are 3 strategies for improving the post-transfer survival of in vitro produced bovine embryos: 1) alter the recipient to improve fertility, 2) identify markers for embryo survival and 3) modify embryo culture to enhance post-culture viability.

Improve Recipient Fertility

McMillan (1998) developed a model to separate the contribution of the embryo and recipient for embryo survival up to day 60 of pregnancy. This model predicted that variation in recipient quality (i.e., the ability of a recipient to carry a pregnancy to term) was a greater source of variation in pregnancy rates after embryo transfer than embryo quality. This suggests that strategies to alter recipient fertility could have a major impact on the survival of in vitro produced bovine embryos.

Despite the importance of the recipient for embryo survival, few studies have been conducted to identify strategies to manipulate recipient fertility to increase the survival of in vitro produced bovine embryos. One strategy that has been evaluated is the use of bovine somatotopin (bST). Administration of bST to lactating dairy cows increases pregnancy rates following artificial insemination (Moreira et al., 2000; Moreira et al., 2001; Santos et al., 2004). In addition, treatment of superovulation donors with bST can increase the percentage of transferable embryos and stimulate embryonic development to the blastocyst stage (Moreira et al, 2002a). Moreover, treatment of lactating recipient cows with bST increased pregnancy rates following the transfer of frozen-thawed in vivo-derived embryos (Moreira et al., 2002a). In contrast, a study in which non-lactating recipients were treated with bST did not affect the survival of in vitro produced bovine embryos (Block et al., 2005). Recent data indicate that bST treatment can be detrimental to embryo survival following artificial insemination if given to non-lactating dairy cows (Bilby et al., 2004). However, despite the beneficial effects of bST on embryo survival in lactating cows, no study evaluating the effect of bST to increase the survival of in vitro produced embryos in lactating cows has been conducted.

The beneficial effects of bST on embryo survival may be mediated by IGF-1, which is increased in the circulation following bST treatment (de la Sota et al., 1993; Bilby et al., 2006).

Another approach to increase levels of IGF-1 in the blood is to feed propylene glycol (Hoedemaker et al., 2004; Formiqoni et al., 1996). In a study in which propylene glycol was administered to heifer recipients for 20 days before embryo transfer, pregnancy rates were increased following the transfer of frozen-thawed embryos produced using superovulation (Hidalgo et al., 2004).

Strategies to regulate the luteolytic cascade have also been put forward as methods for improving pregnancy rates following in vitro embryo transfer. In particular, injection of gonadotropin-releasing hormone (GnRH) at 11-14 days after estrus has been frequently tested for enhancing embryo survival after artificial insemination. The administration of GnRH during this time period can decrease estradiol 17-β secretion (Rettmer et al., 1992; Mann and Lamming, 1995) which could delay luteolysis and thereby allow slowly developing embryos more time to initiate secretion of interferon-τ (IFN- τ). In addition, GnRH can increase progesterone secretion (Rettmer et al., 1992; Mann and Lamming, 1995; Stevenson et al., 1993; Willard et al., 2003) which is important for embryo survival (Mann and Lamming, 1999; Inskeep, 2004) and can be reduced in lactating dairy cows (Sartori et al., 2004). Despite these potential actions, this treatment has only met with limited success (Peters et al., 2000; Franco et al., 2006b). The application of a similar strategy for lactating, in vitro embryo transfer recipients did not affect pregnancy rates (Block et al., 2003; Franco et al., 2006a).

Another molecule that exerts similar to actions as GnRH, is human chorionic gonadotrophin (hCG). Treatment of cows (Santos et al., 2001) and heifers (Diaz et al., 1998) at day 5 of the estrous cycle can cause ovulation of the first wave dominant follicle thereby forming an accessory corpus luteum an increasing plasma concentrations of progesterone. Nishigai and colleagues (2002) reported that administration of hCG at day 6 can increase pregnancy rates

following the transfer of frozen-thawed in vivo-derived embryos. Recently, it was reported that the injection of flunixin meglumine, a non-specific inhibitor of prostaglandin synthesis, on day 15 and day 16 after insemination significantly increased pregnancy rates in heifers (Guzeloglu et al., 2007). Use of flunixin meglumine as a more direct approach to block luteolysis may be beneficial for enhancing the survival of in vitro produced embryos as well.

Identify Markers for Embryo Survival

Another strategy to enhance the post-transfer survival of bovine embryos produced in vitro is to identify markers for embryo survival that can be used to develop non-invasive assays for selecting embryos with an enhanced capacity for survival before to transfer. Currently, the most popular criterion used to select embryos for transfer is morphological assessment (Van Soom et al., 2003). Although embryo quality grades can be predictive of an embryo's ability to survive following transfer (Wright, 1981; Lindner and Wright, 1983; Hasler, 2001), such criteria are subjective (Farin et al., 1995).

Another approach for selecting embryos with enhanced developmental competence is to select the embryos that cleave the fastest after fertilization. In several species, including cattle, fast cleaving embryos are more likely to develop to the blastocyst stage (Lonergan et al., 2006). In some species, such as humans, this criteria can also be used to select embryos that are more competent to survive after transfer (Shoukir et al., 1997). In cattle, however, this criterion is not predictive of embryo survival after transfer. Lonergan and colleages (1999) reported no difference in pregnancy rates between embryos that cleaved by 30 hrs after insemination and embryos that cleaved after 36 hrs.

In mice, the rate of embryonic development is controlled by a gene called preimplantation embryo development or *ped* (Verbanac and Warner, 1981). This gene has also been reported to affect birth rate, birth weight and survival (Warner et al., 1991; Warner et al., 1993). The *ped*

gene is located at the Q region of the mouse major histocompatibility complex (Warner et al., 1987; Warner et al., 1991). Fair and colleagues (2004) have investigated expression of major histocompatibility complex class I transcripts in pre-implantation bovine embryos and reported that embryos that cleaved by 28 hrs post-insemination had an increased relative abundance of class I major histocompatibility complex transcripts compared to embryos that cleaved after 36 hrs. These results suggest that cattle may have a gene with a similar function to the mouse *ped* gene and could be used as a marker for embryo selection. However, further investigation is required to identify the specific gene and its sequence.

Measurement of metabolic activity is another potential strategy to select embryos prior to transfer (Gardner and Lane, 1997; Donnay et al., 1999). In particular the measurement of glucose uptake has been correlated with developmental capacity after transfer. Renard and colleagues (1980) were the first to report an effect of glucose uptake on subsequent post-transfer survival. A retrospective analysis indicated that the glucose uptake of day 10 in vivo produced bovine blastocysts was positively correlated with survival following transfer. A correlation between glucose uptake and embryo survival has also been reported for murine (Gardner and Leese, 1987) and human (Gardner et al., 2001) embryos. In addition to glucose, recent research using a nanorespirometer indicates that embryo respiration may also be an indicator of embryo viability (Lopes et al., 2007). However, further research with more transfers is needed to confirm these results.

The use of proteomics could also provide new insights into novel markers which are important for embryo survival after transfer and that can be measured readily in embryo culture medium (Katz-Jaffe et al., 2006). In humans, a marker associated with pregnancy establishment has been identified. Embryos which secrete the soluble form of human leukocyte antigen-G

(sHLA-G) are associated with increased pregnancy and implantation rates (Fuzzi et al., 2002; Noci et al., 2005; Sher at al., 2005; Desai et al., 2006). Moreover, a commercial ELISA kit that can detect sHLA-G in culture samples has been developed and tested (Desai et al., 2006). In this particular study, females that received at least one embryo that secreted sHLA-G had significantly higher pregnancy and implantation rates compared to females that did not receive any embryos positive for sHLA-G secretion (64% and 38% vs. 36% and 19%, respectively).

In cattle, there are currently no markers or assays that can be used to select embryos based on their capacity to survive after transfer. There is some potential for selecting blastocyst stage embryos based on group II caspase activity (Jousan, 2006). Day 7 bovine blastocyst stage embryos which are classified as having low group II caspase activity are more likely to hatch following culture to day 10 than embryos that are classified as having high group II caspase activity (45.5% vs. 24.5%, respectively). Although this procedure may have promise for selecting embryos for transfer, further research is needed to determine whether group II caspase activity (involved in apoptosis cascade) is predictive of embryo survival in vivo.

Modify Embryo Culture Conditions

As described above, production of bovine embryos in vitro causes several alterations in embryo morphology and physiology which have consequences for survival and development after transfer. Recent studies using the sheep oviduct as a model for in vivo embryo development have demonstrated the significant impact embryo culture conditions can have on embryo developmental characteristics and post-culture viability (Enright et al., 2000; Lazzari et al., 2002; Rizos et al., 2002; Lonergan et al., 2006). Thus, another strategy for improving the survival of in vitro produced embryos following transfer is to modify embryo culture conditions to more closely mimic the microenvironment found in vivo.

. One approach is to modify embryo culture with growth factor and/or cytokine molecules. Many growth factors and cytokines are expressed by the oviduct, uterus and preimplantation (Kane et al., 1997; Diaz-Cueto and Gerton, 2001; Hardy and Spanos, 2002). Furthermore, the embryo itself expresses many of the growth factor and cytokine receptors, suggesting the potential for both autocrine and paracrine regulation of development. The addition of growth factors and cytokines to embryo culture can also have a beneficial effect on several aspects of embryo development, including metabolism, differentiation and apoptosis. Moreover, the supplementation of embryo culture with certain cytokine and growth factor molecules has been reported to increase embryo survival following transfer in mice (Roudebush et al., 1999; Sjoblom et al., 2005) and cattle (Block et al., 2003).

Several growth factor and cytokine molecules have been tested for their effects during bovine embryo culture, including epidermal growth factor (Flood et al., 1993; Keefer et al., 1994; Shamsuddin et al., 1994; Lee and Fukui, 1995; Sirasathien and Brackett, 2003; Sirasathien et al., 2003), fibroblast growth factor (Larson et al., 1992b; Shamsuddin et al., 1994; Lee and Fukui, 1995), granulocyte-macrophage colony stimulating factor (de Moraes et al., 1997), IGF-1 (Herrler et al., 1992; Lee and Fukui et al., 1995; Matsui et al., 1995; Palma et al., 1997; Byrne et al., 2002b; Hernandez-Fonseca et al., 2002; Moreira et al., 2002b; Block et al., 2003; Sirasathien and Brackett, 2003; Sirasathien et al., 2003b; Lima et al., 2006) and IGF-2 (Flood et al., 1993; Shamsuddin et al., 1994; Byrne et al., 2002b), interleukin-1 (Paula-Lopes et al., 1998), leukemia inhibitory factor (Fukui and Matsuyama, 1994; Han et al., 1995; Funstun et al, 1997; Sirasathien et al., 2003a; Vejlsted et al., 2005; Rodriguez et al., 2007), nerve growth factor (Flood et al., 1993), transforming growth factor-α (Flood et al., 1993) and -β (Flood et al., 1993; Keefer et al.,

1994; Lee and Fukui et al., 1995), and platelet derived growth factor (Shamsuddin et al., 1994; Larson et al., 1992a).

Many of these growth factors and cytokines have a beneficial effect on bovine embryo development in vitro. Surprisingly, only two of these molecules, leukemia-inhibitory factor (Sirasathien et al., 2003b) and IGF-1 (Hernandez-Fonseca et al., 2002; Block et al., 2003), have been tested for their effects on subsequent embryo survival following transfer. This review will focus on IGF-1 because its actions on bovine embryo development in vitro have been extensively studied and also because IGF-1 is the only one that can affect embryo survival in vivo, as will be discussed in the subsequent sections.

Insulin-like Growth Factor-1

Biology of IGF-1

Insulin-like growth factor-1 is a single-chain polypeptide that is a member of the IGF family of cell signaling factors. This family also includes another ligand, IGF-2, two cell surface receptors, IGF-1R and IGF-2R, as well as at least 6 IGF-binding proteins (IGFBP; Dupont and Holzenberger, 2003). The liver is the primary source of circulating IGF-1 and growth hormone is the principle regulator of IGF-1 synthesis from this organ. Concentrations of IGF-1 in the blood are 1000 fold higher than other peptide hormones (Dupont and Holzenberger, 2003). This is a result of the binding of IGF-1 by the IGFBP, in particular, IGFBP-3, which along with the acid labile subunit, helps to extend the half-life of IGF-1 in the circulation. In addition to regulating the half-life of IGF-1, IGFBP also regulate its actions in various cells and tissues of the body (Wetterau et al., 1999). While IGF-1 is primarily produced by the liver, several tissues and cells in the body can also secrete IGF-1 (Dupont and Holzenberger, 2003). This includes the female bovine reproductive tract. Both the oviduct (Schmidt et al., 1994; Pushpakumara et al.,

2002) and the uterus (Geisert et al., 1991; Robinson et al., 2000) express IGF-1 during the stages of preimplantation development.

The actions of IGF-1 are mediated by the IGF-1R receptor, which is a heterotetrameric glycoprotein and member of the receptor tyrosine kinase family of cell surface receptors (Siddle et al., 2001; Dupont and Holzenberger, 2003). Bovine preimplantation embryos express the IGF-1R throughout preimplantation embryo development from the 2-cell stage through the blastocyst stage (Yoshida et al., 1998). Binding of IGF-1 causes autophosphorylation of the IGF-1R which leads to the phosphorylation of tyrosine residues on several docking proteins, including insulin receptor substrate and Shc-homology protein. The phosphorylation of these intracellular substrates then activates one of two major signalling pathways, the phosphatidyl inositol 3' kinase/ Akt pathway or the ras/raf/MAP kinase pathway (Dupont and Holzenberger, 2003). Recent data indicate that both of these pathways are active in bovine preimplantation embryos and help to regulate the anti-apoptotic and proliferative actions of IGF-1 (Jousan and Hansen, 2007).

Actions of IGF-1 on Bovine Embryo Development and Survival in Vivo

The use of knock-out models in mice have indicated that IGF-1 is not required for preimplantation embryo development because mice that have a null mutation for IGF-1R are capable of producing offspring (Liu et al., 1993). However, these experiments do indicate that IGF-1 is important for normal development as offspring from IGF-1R null mice are 45% of normal size and die shortly after birth (Liu et al., 1993).

Even though such models are not possible in cattle, there are data to support a relationship between IGF-1 and embryo development and survival in vivo. As mentioned previously, administration of bST to lactating dairy cows, which increases plasma concentrations of IGF-1 (de la Sota et al., 1993; Bilby et al., 2006), improves pregnancy rates following timed artificial

insemination (Moreira et al., 2000; Moreira et al., 2001; Santos et al., 2004). In addition, treatment of donor animals with bST decreased the number of unfertilized ova, increased the percentage of transferable embryos, and stimulated embryonic development to the blastocyst stage following superovulation (Moreira et al., 2002a). Moreover, the embryos produced from donors treated with bST were more likely to survive following transfer to lactating dairy cows than embryos from control cows.

Recent studies indicate that bST treatment of lactating dairy cows can increase the proportion of conceptuses recovered at day 17 of gestation (Bilby et al., 2006). Treatment with bST also increased conceptus length and total interferon-τ in uterine flushings suggesting that bST treatment may increase pregnancy rates by improving the capacity of conceptuses to block luteolysis.

In contrast to the effects of bST on embryo development and survival in lactating cows, actions of bST are not beneficial in non-lactating cows or heifers. In one study, treatment of heifers with bST at the time of transfer of either in vitro or in vivo produced embryos did not affect pregnancy rates (Hasler et al., 2002). Similarly, treatment of non-lactating cows with bST on the day of anticipated ovulation did not affect pregnancy rates following the transfer of in vitro produced embryos (Block et al., 2005). In addition, non-lactating cows that were treated with bST and artificially inseminated had a lower proportion of recovered conceptuses on day 17 of gestation than for control cows.

One explanation for the discrepancy between non-lactating animals and lactating cows in terms of their response to bST is differences in circulating IGF-1 concentrations. Non-lactating cows have higher concentrations of plasma IGF-1 than do lactating cows (213 vs 150 ng/mL; de la Sota et al., 1993). Treatment of lactating cows with bST increases plasma IGF-1 to 306

ng/mL while bST treatment of non-lactating cows increased plasma IGF-1 to 458 ng/mL (de la Sota et al., 1993). It may be that bST treatment of lactating cows increases IGF-1 concentrations to a more optimal level for embryo survival while bST treatment of non-lactating animals increases IGF-1 to a level that is too high and therefore detrimental to embryo survival. Such a possibility is supported by data in humans and mice. Women with polycystic ovary syndrome have elevated insulin and IGF-1 concentrations and experience higher pregnancy losses (Tulppala et al., 1993; Sagle et al., 1988). Moreover, high IGF-1 concentrations during in vitro culture of murine embryos resulted in a decrease in implantation rate following transfer (Pinto et al., 2002.)

When evaluating the effects of bST on embryo survival in vivo, it is not possible to separate the actions of IGF-1 from bST. However, another treatment which can increase plasma concentrations of IGF-1 is propylene glycol (Hoedemaker et al., 2004; Formiqoni et al., 1996). In a study in which propylene glycol was administered orally to heifer recipients for 20 days before embryo transfer, pregnancy rates were increased following the transfer of frozen-thawed embryos produced using superovulation (Hidalgo et al., 2004). This result also suggests a role for IGF-1 in embryo development and survival in vivo.

Actions of IGF-1 on Bovine Embryo Development in Vitro

Addition of IGF-1 to culture medium can have many effects on embryonic development. Addition of IGF-1 at concentrations ranging from 2 to 200 ng//mL have been reported to increase the proportion of embryos becoming morulae at day 5 post-insemination (Matsui et al., 1995, Matsui et al., 1997) and blastocysts between day 6.5 and 8 after insemination (Herrler et al., 1992; Palma et al., 1997; Prelle et al., 2001; Byrne et al., 2002b; Moreira et al., 2002b; Block et al., 2003; Sirisathien and Brackett, 2003; Sirisathien et al., 2003c; Lima et al., 2006). In addition, IGF-1 treatment can increase the proportion of embryos that develop to advanced

stages of blastocyst development (expanded and hatched) at day 8 after fertilization (Moreira et al., 2002b; Block et al., 2003). Actions of IGF-1 on embryo development appear to be mediated by the IGF-1 receptor because addition of a monoclonal antibody specific for the α subunit of the IGF-1R blocked the actions of IGF-1 on embryo development to the morula stage (Matsui et al., 1997).

In addition to promoting embryo development, IGF-1 can have mitogenic actions on bovine embryos. Addition of IGF-1 has been reported to increase blastocyst cell number in several studies (Prelle et al., 2001; Byrne et al., 2002b; Makaravich and Marrkula, 2002; Moreira et al., 2002b; Sirisathien and Brackett, 2003; Sirisathien et al., 2003c). In some reports, the increase in cell number has bee attributed to actions of IGF-1 on the trophoblast cells (Prelle et a., 2001; Makarevich and Markkula, 2002) and in another report IGF-1 increased the number of inner cell mass cells (Sirisathien et al., 2003c). One reason for the increased cell number in IGF-1 treated embryos may be related to actions of IGF-1 cell survival. Embryos cultured in the presence of IGF-1 have a reduced proportion of apoptotic blastomeres (Byrne et al., 2002b; Sirisathien and Brackett, 2003).

Recent research indicates that IGF-1 can act as a survival factor for the bovine preimplantation embryo exposed to heat shock (Jousan and Hansen, 2004; 2007). While heat shock of day 5 embryos increased apoptosis and reduced development to the blastocyst stage at day 8 after fertilization, treatment of embryos with IGF-1 blocked the induction of apoptosis and reduced the decrease in development caused by heat shock (Jousan and Hansen, 2004, 2007). These studies have also demonstrated that the anti-apoptotic actions of IGF-1 require the phosphatidyl inositol 3' kinase pathway while the proliferative actions of IGF-1 require the mitogen activated protein kinase pathway (Jousan and Hansen, 2007). Interestingly the anti-

apoptotic actions of IGF-1 are not required to for IGF-1 to block effects of heat shock on development to the blastocyst stage after heat shock (Jousan and Hansen, 2007).

Hernandez and Fonseca (2002) were the first researchers to test whether addition of IGF-1 to embryo culture could affect subsequent embryo survival following transfer to recipients. Their results indicated that there was no effect of IGF-1 on the survival of frozen-thawed in vitro produced embryos. However, only 10 recipients were used per treatment group and these low numbers severely limit the conclusions that can be derived from this study. In a more recent field trial (Block et al., 2003) in which more than 200 heat-stressed lactating Holstein cows were used as recipients, IGF-1 treatment increased pregnancy rates at day 53 and day 81. In addition, recipients that received IGF-1 treated embryos had an increased proportion of viable calves and IGF-1 had no effect on calf birth weights or sex ratio.

Questions for Dissertation

While supplementation of embryo culture medium with IGF-1 can increase pregnancy and calving rates following the transfer of embryos to heat-stressed, lactating dairy cows, several questions remain unanswered: The first is what actions does IGF-1 exert during embryo development in vitro that allows for increased embryo survival after transfer? As described above, it is well recognized that IGF-1 can have many effect on embryo development in vitro. However, despite all of the research related to the effects of IGF-1 on embryo development, cell number and apoptosis, no study has analyzed the effects of IGF-1 on gene expression in bovine blastocysts. Since culture conditions can affect gene expression in bovine embryos, it may be possible that IGF-1 acts to improve embryo survival by altering the abundance of certain genes. The second question is what actions does IGF-1 have on post-transfer embryo development that allow for improved embryo survival? It is hypothesized that one action of IGF-1 is to increase conceptus length and IFN-τ in the uterus around the time of maternal recognition of pregnancy.

The basis of this hypothesis is that similar actions are caused by bST (Bilby et al., 2006) and the supposition that these bST effects are mediated by IGF-1. Actions of IGF-1 on conceptus length and IFN-τ secretion could enhance the capacity of embryos to block luteolysis and thereby promote embryonic survival. The final question to be addressed in this thesis is whether effects of IGF-1 on embryo survival are a general effect of IGF-1 or one specific to heat stress. Since IGF-1 treatment in vitro can reduce the deleterious effects of heat shock on apoptosis and embryo development (Jousan and Hansen, 2004; Jousan and Hansen, 2006), and since the experiment by Block and others (2003) showing an effect of IGF-1 on post-transfer survival was conducted in the summer, it is possible that actions of IGF-1 on embryo survival are the result of a thermoprotective effect of IGF-1. If so, one would not see beneficial effects of IGF-1 on embryo survival for recipients receiving embryos during cool periods.

These questions form the basis for this dissertation. Subsequent chapters will address each of these in order and the General Discussion in Chapter 5 will provide an overview of findings and an updated perspective of how IGF-1 affects embryo physiology to affect post-transfer survival.

CHAPTER 2
EFFECT OF INSULIN-LIKE GROWTH FACTOR-1 ON CELLULAR AND MOLECULAR CHARACTERISTICS OF BOVINE BLASTOCYSTS PRODUCED IN VITRO

Introduction

The production of bovine embryos in vitro is associated with altered metabolism (Khurana and Niemann, 2000), gene expression (Bertolini et al., 2002a; Lazzari et al., 2002; Lonergan et al., 2003), and cryo-survival (Enright et al., 2000; Rizos et al., 2002) compared to embryos produced following superovulation. Differences are also manifested during post-culture development in that the transfer of in vitro produced embryos is associated with reduced embryo survival (Farin and Farin, 1995; Hasler, 1995; Drost et al., 1999), fetal and neonatal overgrowth (Lazzari et al., 2002) and increased fetal and placental abnormalities (van Wagtendonk-de Leeuw et al., 1998, 2000; Farin et al., 2006).

The addition of growth factors to culture medium is one potential strategy to improve embryo development and survival. In vivo, the oviduct, uterus and the early developing embryo express an array of growth factors including epidermal growth factor, IGF-1, IGF-2, platelet derived growth factor, transforming growth factor-α, and fibroblast growth factor (Kane et al., 1997; Díaz-Cueto and Gerton, 2001; Yaseen et al., 2001; Hardy and Spanos, 2002). Moreover, in many cases, the embryo has been shown to express the receptor for these growth factors so that these molecules may exert paracrine and autocrine functions in early embryo development.

One of the most studied growth factors is IGF-1. Insulin-like growth factor-1 can affect bovine embryo development in vitro in several ways. Addition of IGF-1 to culture has been reported to stimulate development of bovine embryos to the blastocyst stage

(Herrler et al., 1993; Palma et al., 1997; Prelle et al. 2001; Moreira et al., 2002b; Sirisathien et al., 2003; Block et al., 2003), increase blastocyst cell number (Byrne et al., 2002b; Moreira et al., 2002; Sirisathien and Brackett, 2003; Sirisathien et al., 2003b) and glucose transport (Pantaleon and Kaye, 1996), and reduce the proportion of blastomeres that are apoptotic (Byrne et al., 2002b; Markkula and Makarevich, 2002; Sirisathien and Brackett, 2003). Moreover, treatment of embryos during culture with IGF-1 increases post-transfer survival of those embryos when transferred into heat stressed, lactating dairy cows (Block et al., 2003; Chapter 4).

The objective of the present experiment was to determine molecular and cellular actions of IGF-1 that could explain the increased potential for embryonic survival after transfer (Block et al., 2003; Chapter 4). Focus was placed on effects of IGF-1 on cell number, cell allocation, and apoptosis and the relative abundance of several developmentally important mRNA transcripts.

Materials and Methods

All materials were purchased from Sigma (St. Louis, MO) or Fisher Scientific (Fairlawn, NJ) unless specified otherwise.

Culture Media

Sperm-Tyrode's Lactate, IVF-Tyrode's Lactate, and Hepes Tyrode's Lactate were purchased from Caisson Laboratories, Inc. (Logan, UT). These media were used to prepare Sperm-Tyrode's Albumin Lactate Pyruvate (TALP), IVF-TALP, and Hepes-TALP as described previously (Parrish et al., 1986). Oocyte collection medium (OCM) was Tissue Culture Medium-199 (TCM-199) with Hank's salts without phenol red (Atlanta Biologicals, Norcross, GA) and supplemented with 2% (v/v) bovine steer serum (Pel-Freez, Rogers, AR), 2 U/mL heparin, 100 U/mL penicillin-G, 0.1 mg/mL

streptomycin, and 1 mM glutamine. Oocyte maturation medium (OMM) was TCM-199 (Invitrogen, Carlsbad, CA) with Earle's salts supplemented with 10% (v/v) bovine steer serum, 2 μg/mL estradiol 17-β, 20 μg/mL bovine follicle stimulating hormone (FSH; Folltropin-V; Bioniche, Belleview, Ontario, Canada), 22 μg/mL sodium pyruvate, 50 μg/mL gentamicin sulfate, and 1 mM glutamine. Potassium simplex optimized medium (KSOM) that contained 1 mg/ml BSA was from Caisson. On the day of use, KSOM was modified to produce KSOM-BE2 as described previously (Soto et al., 2003).

In Vitro Embryo Production

Embryos were produced in vitro as described previously (Soto et al., 2003). Briefly, cumulus-oocyte complexes (COCs) were obtained by slicing 2- to 10-mm follicles on the surface of ovaries (predominantly beef cattle) obtained from Central Beef Packing Co. (Center Hill, FL). Those COCs with multiple layers of compact cumulus cells were washed two times in OCM and used for subsequent steps. Groups of 10 COCs were placed in 50-μl drops of OMM overlaid with mineral oil and matured for 21-24 h at 38.5°C in an atmosphere of 5% (v/v) CO_2 in humidified air. Matured COCs were then washed once in HEPES-TALP and transferred in groups of 30 to 4-well plates containing 600 μl of IVF-TALP and 25 μl of PHE (0.5 mM penicillamine, 0.25 mM hypotaurine, and 25 μM epinephrine in 0.9% [w/v] NaCl) per well and fertilized with ~1 x 10^6 Percoll-purified (Amersham Pharmacia Biotech, Uppsala, Sweden) spermatozoa from a pool of frozen-thawed semen from three bulls of various breeds. A different pool of semen was used for each replicate. Depending on the experiment, COCs and spermatozoa were allowed to coincubate for 20-24 h at 38.5°C in an atmosphere of 5% (v/v) CO_2 in humidified air. After fertilization, putative zygotes were removed from fertilization wells, denuded of cumulus cells by vortex mixing in HEPES-TALP containing 1000

U/ml hyaluronidase, and randomly placed in groups of 25 in 50-µl drops of either KSOM-BE2 or KSOM-BE2 containing 100 ng/mL IGF-1 (Upstate Biotech, Lake Placid, NY) as described previously (Block et al., 2003). All drops of embryos were overlaid with mineral oil and cultured at 38.5°C in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2. The proportion of cleaved oocytes was recorded on d 3 after insemination and the proportion of blastocysts and advanced blastocysts was recorded on day 7.

TUNEL Assay

The TUNEL assay was performed as described previously (Jousan and Hansen, 2004) using an in situ cell death detection kit (Roche, Indianapolis, IN). Embryos were removed from culture and washed two times in 50-µl drops of 10 mM KPO_4 pH 7.4 containing 0.9% (w/v) NaCl (PBS) and 1 mg/ml polyvinylpyrrolidone (PVP; Eastman Kodak, Rochester, NY; PBS-PVP). Zona pellucida-intact embryos were fixed in a 50-µl drop of 4% (w/v) paraformaldehyde in PBS for 15 min at room temperature, washed twice in PBS-PVP, and stored in 500 µl of PBS-PVP at 4°C until the time of assay.

On the day of the TUNEL assay, embryos were transferred to a 50-µl drop of PBS-PVP and then permeabilized in 0.1% (v/v) Triton X-100 containing 0.1% (w/v) sodium citrate for 10 min at room temperature. Controls for the TUNEL assay were incubated in 50 µl of RQ1 RNase-free DNase (50 U/ml; Promega, Madison, WI) at 37°C in the dark for 1 h. Positive controls and treated embryos were washed in PBS-PVP and incubated with 25 µl of TUNEL reaction mixture (containing fluorescein isothiocyanate-conjugated dUTP and the enzyme terminal deoxynucleotidyl transferase as prepared by and following the guidelines of the manufacturer) for 1 h at 37°C in the dark. Negative controls were incubated in the absence of terminal deoxynucleotidyl transferase. Embryos were then washed three times in PBS-PVP and incubated in a 25-µl drop of Hoechst

33258 (1 μg/ml) for 15 min in the dark. Embryos were washed three times in PBS-PVP to remove excess Hoechst 33258, mounted on 10% (w/v) poly-L-lysine coated slides using 3- to 4-μl drops of glycerol, and the slides affixed with coverslips. Labeling of TUNEL and Hoechst 33258 nuclei was observed using a Zeiss Axioplan 2 epifluorescence microscope (Zeiss, Göttingen, Germany). Each embryo was analyzed for total cell number (blue nuclei) and TUNEL-positive blastomeres (green nuclei) with DAPI and FITC filters, respectively, using a 20x objective.

Differential Staining

Zona-intact embryos were removed from culture and washed 3 times in 50 μL drops of PBS-PVP. To label trophectoderm cells (TE), embryos were placed into 500 μL of PBS-PVP containing 0.5% Triton X-100 and 100 μg/mL propidium iodide for 30 s at 37ºC. Embryos were then washed immediately through 3 wells of a 4-well plate containing 500 μL of PBS-PVP each. To fix embryos and stain inner cell mass cells (ICM), embryos were then incubated in a 50 μL drop of PBS-PVP containing 4% paraformaldehyde and 10 μg/mL Hoechst 33258 for 15 min at room temperature. Embryos were then washed three times in PBS-PVP, mounted on 10% (w/v) poly-L-lysine coated slides using 3- to 4-μl drops of glycerol, and then covered with coverslips. Labeling of propidium iodide and Hoechst 33258 nuclei was observed using a Zeiss Axioplan 2 epifluorescence microscope (Zeiss, Göttingen, Germany). Each embryo was analyzed for the number of ICM (blue nuclei), the number of TE cells (pink nuclei), and total cell number (blue + pink nuclei) with a DAPI filter using a 20x objective.

RT-PCR

The relative abundance of 14 gene transcripts was determined using semi-quantitative RT-PCR as described previously (Wrenzycki et al., 2001b). Primer

sequences, annealing temperatures, fragment sizes, and references for sequences are summarized in Table 2-1. The PCR primers were designed from the coding regions of each gene sequence using the OLIGO program National Biosciences, Plymouth, USA

Harvested embryos were washed 3 times in PBS-PVP and then stored at -80ºC until further processing. Poly(A)$^+$ RNA was isolated from single blastocysts as previously described (Schultz et al., 1996Wrenzycki et al., 1999) and was used immediately for reverse transcription (RT) that was carried out in a total volume of 20 µl using 2.5 µM random hexamers (GeneAmp® RNA PCR Kit components, Applied Biosystems, CA, USAPerkin-Elmer, Wellesley, MA). Prior to RNA isolation, 1 pg of rabbit globin RNA (BRL, Gaithersburg, MD) was added as an external standard. The reaction mixture consisted of 1x RT buffer (50 mM KCl, 20 mM Tris-HCl, pH 8.4; Invitrogen10 mM Tris-HCl, pH 8.3; Perkin-Elmer), 5 mM $MgCl_2$ (Invitrogen), 1 mM of each dNTP (Amersham, Brunswick, Germany), 20 IU RNase inhibitor (GeneAmp® RNA PCR Kit components, Applied Biosystems, CA, USAPerkin-Elmer), and 50 IU murine leukemia virus reverse transcriptase (GeneAmp® RNA PCR Kit components, Applied Biosystems, CA, USAPerkin-Elmer). The mixture was overlaid with mineral oil to prevent evaporation. The RT reaction was carried out at 25°C for 10 min, 42°C for 1 h followed by a denaturation step at 99°C for 5 min and flash cooling on ice. Polymerase chain reaction (PCR) was performed with cDNA equivalents as described in Table 1 from individual embryos as well as 50 fg of globin RNA in a final volume of 50 µl of 1x PCR buffer (50 mM KCl, 20 mM Tris-HCl pH 8.4; Invitrogen, Karlsruhe, Germany20 mM Tris-HCl, pH 8.4, 50 mM KCl; Gibco BRL, Eggenstein, Germany), 1.5 mM $MgCl_2$ (Invitrogen, Karlsruhe, Germany),, 200 µM of each dNTP, 1 0.5 µM of each sequence-

specific primer (globin: 0.5 μM). The PCR reactions were performed using a PTC-200 thermocycler (MJ Research, Watertown, MA). To ensure specific amplification, a hot start PCR was employed by adding 1 IU Taq DNA polymerase (Invitrogen, Karlsruhe, Germany)Gibco) at 72°C. The PCR program employed an initial step of 97°C for 2 min and 72°C for 2 min (hot start) followed by different cycle numbers (see Table 1) of 15 sec each at 95°C for DNA denaturation, 15 sec at different temperatures for annealing of primers, and 15 sec at 72°C for primer extension. The last cycle was followed by a 5-min extension at 72°C and cooling to 4°C. As negative controls, tubes were prepared in which RNA or reverse transcriptase was omitted during the RT reaction.

The RT-PCR products were subjected to electrophoresis on a 2% (w/v) agarose gel in 1x TBE buffer (90 mM Tris, 90 mM borate, 2 mM EDTA, pH 8.3) containing 0.2 μg/ml ethidium bromide. The image of each gel was recorded using a charge-coupled device camera (Quantix, Photometrics, München, Germany) and the IP Lab Spectrum program (Signal Analytics Corporation, Vienna, VA). The intensity of each band was assessed by densitometry using an image analysis program (IP Lab Gel). The relative amount of the mRNA of interest was calculated by dividing the intensity of the band for each transcript by the intensity of the globin band for each embryo. To circumvent the problem that the differences in the relative abundance of the transcripts were due to different cell numbers of the blastocysts analyzed, the relative abundance of each transcript for each embryo was divided by the mean total cell number for that treatment and multiplied by 100. The value for mean total cell number for embryos in the replicates used for RNA analysis were 131.8 cells (n=96) for control embryos and 117.7 cells (n = 76) for control embryos. For each pair of gene-specific primers, semilog plots of the

fragment intensity as a function of cycle number were used to determine the range of cycle number over which linear amplification occurred and the number of PCR cycles was kept within this range (Wrenzycki et al., 1999). Because the total efficiency of amplification for each set of primers during each cycle is not known, such an assay can only be used to compare relative abundances of one mRNA among different samples (Temeles et al., 1994).

Experiment 1: Effect of IGF-1 on Total Cell Number, Apoptosis and Cell Allocation

Grade 1 expanded blastocysts (Robertson and Nelson, 1998) were harvested on day 7 after fertilization. For 7 replicates, harvested embryos were used to determine the proportion of apoptotic nuclei with the TUNEL assay. There were between 71 and 84 embryos for each treatment. For an additional 7 replicates, harvested embryos were used to determine cell allocation to the ICM and TE using differential staining. There were between 146 and 163 embryos for each treatment. For all 14 replicates, harvested embryos were used to evaluate total cell number.

Experiment 2: Effect of IGF-1 on the Relative Abundance of Developmentally Important Genes

Grade 1 expanded blastocysts (Robertson and Nelson, 1998) were harvested on day 7 after insemination. Approximately half of the selected embryos (Control n = 104 and IGF-1 n = 93) were then randomly assigned to evaluate the relative abundance of mRNA transcripts for IGF-1 receptor (IGF-1R), IGF binding protein-2 (IGFBP2), IGF binding protein-2 (IGFBP3), IGF binding protein-5 (IGFBP5), glucose transporter-1 (Glut1), Glut3, Glut8, heat shock protein 70.1 (Hsp70), Bax, Bcl, desmocollin-II (Dc II), E-cadherin (Ecad) and plakophilin (Plako). The remaining embryos (Control n = 96 and

IGF-1 n = 76) were used to determine total cell number. A total of 4 replicates were completed.

Statistical Analysis

Data were analyzed by analysis of variance using the GLM procedure of SAS (SAS for Windows, version 9.0, SAS Inst., Inc., Cary, NC). Percentage data were transformed by arcsin transformation before analysis. Independent variable for the following variables were IGF-1 treatment and replicate: cleavage rate, blastocyst development, total cell number, percent apoptosis, the number of ICM and TE cells, and the ratio of TE cells to ICM cells. For gene transcripts, treatment was the only independent variable included in the model. All values reported are least-squares means ± SEM. Probability values for percentage data are based on analysis of arcsin-transformed data while least-squares means are from analysis of untransformed data.

Results

Among grade 1 expanded blastocysts selected on d 7 after fertilization, treatment with IGF-1did not affect total cell number or the proportion of blastomeres that were apoptotic (Table 2). There was also no effect of IGF-1 treatment on the number of cells in the TE or the ratio of TE:ICM. There was, however, a tendency ($P < 0.06$) for IGF-1 treated embryos to have less cells in the ICM than controls (Table 2-2).

Results on relative abundance of the 14 gene transcripts are presented in Figure 1. Among transcripts involved in cell to cell adhesion and blastocyst expansion, treatment with IGF-1 tended ($P < 0.08$) to increase relative abundance of NaK transcripts and increased ($P < 0.01$) relative abundance of Dc II transcripts. There was no effect of IGF-1 on relative abundance of transcripts for Ecad or Plako. Of the two genes examined that are involved in apoptosis, IGF-1 tended to increase ($P < 0.06$) relative abundance of Bax

transcripts and had no effect on amounts of Bcl transcript. In addition, IGF-1 treatment reduced ($P < 0.05$) the relative abundance of Hsp70 transcripts. For transcripts of genes involved with insulin-like growth factor, IGF-1 tended ($P < 0.07$) to reduce abundance of IGF1R mRNA and increased ($P < 0.02$) abundance of IGFBP3 transcripts. There was no effect of IGF-1 treatment on the relative abundance of transcripts for IGFBP2 and IGFBP5. There was also no effect of IGF-1 on the relative abundance of Glut1, Glut3 or Glut8 mRNA.

Discussion

Insulin-like growth factor-1 can change the physiology of the bovine embryo so that, at least under some conditions, it is more likely to complete development to the blastocyst stage (Palma et al., 1997; Prelle et al. 2001; Moreira et al., 2002b; Sirisathien et al., 2003b, Block et al., 2003) and have greater likelihood of establishing pregnancy when transferred to recipients (Block et al., 2003; Chapter 4). Current results indicate that among the changes in embryo physiology caused by IGF-1 at the blastocyst stage are increases in the relative abundance of transcripts for Dc II, Na/K, and Bax and IGFBP3 and a decrease in amounts of Hsp70 transcripts. In contrast, there was no effect of IGF-1 treatment on cell number, allocation to the ICM and TE, or the proportion of blastomeres undergoing apoptosis. Thus, effects of IGF-1 on subsequent survival in vivo are more likely the result of differences in gene expression rather than in changes in cell number, allocation or apoptosis.

Among the transcripts elevated by IGF-1 were Dc II and Na/K. Both of these genes are involved with blastocyst formation. Desmocollin II is involved in the formation of desmosomes and these play a critical role in stabilizing the TE during blastocyst formation and expansion (Fleming et al., 1991; Collins et al., 1995). In

addition, Na/K regulates the accumulation of fluid in the blastoceole (Watson and Barcroft, 2001) as well as the formation of tight junctions during blastocyst expansion (Violette et al., 2006). Such differences in mRNA for Dc II and Na/K may indicate that IGF-1 treated embryos were at a more advanced stage of blastocyst expansion than controls even though all embryos were similar in terms of gross morphology. In addition, IGF-1 treated embryos may possess a more effective TE with respect to ion and water movement.

Compared to embryos produced following superovulation, embryos produced in vitro under sub-optimal culture conditions have an increased abundance of Hsp70 mRNA (Wrenzycki et al., 2001a; Lazzari et al., 2002; Sagirkaya et al., 2006). In the present study, IGF-1 reduced Hsp70 transcript abundance. One possibility for this finding is that IGF-1 makes embryos more resistant to one or more stresses associated with culture. Treatment of cultured embryos with IGF-1 reduced the effect of hydrogen peroxide (Kurzawa et al., 2002) and heat shock (Jousan and Hansen, 2004, 2006).

One of the actions of Hsp70 is to block apoptosis (Garrido et al., 2001, 2003). The fact that Hsp70 transcripts were reduced by IGF-1 implies that effects on Hsp70 synthesis are not involved in the anti-apoptotic effects of IGF-1 on apoptosis induced spontaneously during culture (Herrler et al., 1998; Lighten et al., 1998; Byrne et al., 2002a; Fabian et al., 2004) or by ultraviolet radiation (Herrler et al., 1998), tumor necrosis factor – α (Byrne et al., 2002a), or heat shock (Jousan and Hansen, 2004). There was also no effect of IGF-1 on transcript abundance for the anti-apoptotic gene, Bcl. Moreover, relative abundance of transcripts for the pro-apoptotic gene Bax was increased by IGF-1. This is somewhat surprising given that increased abundance of Bax might

make embryos more susceptible to apoptosis. In addition, IGF-1 reduced abundance of the Bax gene transcript in porcine embryos (Kim et al., 2006). The increased abundance of Bax coupled with no effect of IGF-1 treatment on the abundance of the anti-apoptotic gene Bcl may help to explain why IGF-1 treated embryos in the present study did not have reduced levels of apoptosis.

The IGFBPs regulate the activity of IGF-1 in several ways, including extending the half-life of IGF-1, transporting and localizing IGF-1 to specific cell types and tissues, and stimulating and/or inhibiting IGF-1 actions at the cellular level (Jones and Clemmons, 1995; Clemmons, 1997; Cohick, 1998; Mohan and Baylink, 2002). While the precise role of IGFBPs in early embryo development is not fully understood, IGF-1 can alter the expression of IGFBPs by the early embryo (Prelle et al., 2001) and IGFBPs can modulate the effects of IGF-1 on early embryo development (Lin et al,. 2003). In the present study, IGF-1 treatment increased the abundance of IGFBP3 transcripts. The majority of IGF-1 in the circulation is bound by IGFBP3 (Jones and Clemmons, 1995) and IGF-1 has been reported to increase circulating levels of IGFBP3 in vivo (Zapf et al., 1989; Camancho-Hubner et al., 1991a; Liao et al., 2006) as well as mRNA and protein levels in vitro (Bale and Conover, 1992; Camancho-Hubner et al., 1991b; Fleming et al., 2005). Treatment with IGF-1 also reduced transcripts for IGF1R, as has been found previously for bovine embryos (Prelle et al., 2001) and other cells (Hernandez-Sanchez et al., 1997). Taken together, it appears that one of the embryonic responses to IGF-1 is to dampen embryonic responses to IGF-1 through increased sequestration (via IGFBP3) and receptor downregulation.

The present finding that IGF-1 did not affect the proportion of embryos that became blastocysts in culture is in contradiction of studies from our laboratory (Moreira et al., 2002b; Block et al., 2003; Chapter 4) and others (Palma et al., 1997; Prelle et al. 2001; Sirisathien et al., 2003b) that IGF-1 causes an increase in the proportion of embryos that reach the blastocyst stage. Differences between the present study and others may be related to differences in culture conditions because these have been reported to affect whether IGF-1 stimulates embryo development (Herrler et al., 1992; Palma et al., 1997). It may be that IGF-1 is more effective at increasing blastocyst development when the culture system results in a low yield of blastocysts. In the present study, the proportion of oocytes that developed to the blastocyst stage in the control group on d 7 was quite high (27.9 ± 1.3%). In previous reports where IGF-1 stimulated embryo development, blastocyst development in the control groups ranged between 9 and 19% on d 7 (Byrne et al., 2002a; Block et al., 2003; block and Hansen, 2007) and between 10.5 and 28.5% on d 8 (Moreira et al., 2002b; Block et al., 2003; Sirisathien et al., 2003b).

Addition of IGF-1 to embryo culture in the present study did not affect total cell number, the allocation of cells to the ICM and TE, or the percent of blastomeres that were apoptotic. The literature is inconsistent regarding effects of IGF-1 on these characteristics in bovine embryos. Some reports indicate IGF-1 can increase cell number (Byrne et al., 2002b; Moreira et al., 2002b; Sirisathien and Bracket, 2003; Sirisathien et al., 2003b), increase the number of cells in the ICM (Sirisathien et al., 2003) and decrease the percent of blastomeres that were apoptotic (Byrne et al., 2002b; Sirisathien and Brackett, 2003). However, Sirisathien and Brackett (2003) reported a positive effect of

IGF-1 on cell number and apoptosis for embryos collected at d 8 but not for embryos collected at d 7. In addition, Prelle and colleagues (2001) reported no effect of IGF-1 on total cell number or cell allocation to the ICM and TE. Again, culture conditions or timing of development may dictate the nature of the effect of IGF-1 on these characteristics of blastocysts.

In conclusion, treatment of cultured bovine embryos with IGF-1 increased or tended to increase the relative abundance of certain mRNA transcripts, including Na/K, Dc II, Bax, and IGFBP3, and decreased or tended to decrease transcripts for Hsp70 and IGF1R. There was no effect of IGF-1 on the proportion of embryos developing to the blastocyst stage, cell number, cell allocation, or apoptosis. The alteration of steady state levels of certain gene transcripts by IGF-1 treatment may be important for the improved survival of IGF-1 treated embryos reported previously (Block et al., 2003; Block and Hansen, 2007). An increase in Dc II and Na/K may improve blastocyst expansion and development after hatching. Homologous recombination experiments in mice indicate that Dc III, another member of the desmocollin family, is required for preimplantation development (Den et al., 2006). The reduced abundance of Hsp70 transcripts is consistent with the idea that IGF-1 reduced cellular stress and such an effect could also contribute to higher survival.

Table 2-1. Primers used for RT-PCR.

Genes	Primer sequences and positions	Annealing temp (ºC) x cycle number and embryo equivalent	Fragment size (bp)	EMBL accession no.
Glucose transporter 1(Glut1) SLC2A1	5´primer: (894-914) = CAG GAG ATG AAG GAG GAG AGC 3´primer: (1131-1151) = CAC AAA TAG CGA CAC GAC AGT	59 °C x 32 0.05	257	M60448
Glucose transporter 3 (Glut3) SLC2A3	5´primer: (1095-1118) = CCT TGG AGG GAT GGC TTT TTG TTC 3´primer: = CGT GGC TGA GGG GAA GAG CAG TCC	59 °C x 32 0.1	259	NM_174603
Glucose transporter 8 (Glut8) SLC2A8	5´primer: (711-730) = CCT CGC TTC CTG CTG TCT CA 3´primer: (935-954) = CCT CCT CAA AGA TGG TCT CC	58 °C x 34 0.2	244	AY208940.1
Bax	5´primer: (227-249) = TGC AGA GGA TGA TCG CAG CTG TG 3´primer: (402-424) = CCA ATG TCC AGC CCA TGA TGG TC	60°C x 32 0.1	197	NM_173894.1
BCL-xL (Bcl)	5´primer: (197-221) = ATG GAG CCA CTG GCC ACA GCA GAA G 3´primer: (479-503) = GTT GCG ATC CGA CTC ACC AAT ACC T	60°C x 32 0.2	307	NM_001077486
HSP70.1 (Hsp70)	5´primer: (844-864) = GGG GAG GAC TTC GAC AAC AGG 3´primer: (1068-1088) = CGG AAC AGG TCG GAG CAC AGC	60°C x 32 0.2	245	NM_174550.1

Table 2-1. Continued.

Genes	Primer sequences and positions	Annealing temp (ºC) x cycle number and embryo equivalent	Fragment size (bp)	EMBL accession no.
Na+/K+ATPase (NaK) ATPA1	5´primer: (2884-2905) = ACC TGT TGG GCA TCC GAG AGA C 3´primer: (3198-3219) = AGG GGA AGG CAC AGA ACC ACC A	58 °C x 31 0.1	336	NM_001076798
E-cadherin (Ecad) CDH1	5´primer: (1486-1515) = CTC AAG CTC GCG GAT AAC CAG AAC AAA GAC 3´primer: (1785-1814) = AGG CCC CTG TGC AGC TGG CTC AAA TCA AAG	56 °C x 34 0.2	332	X06339
Desmocollin 2 (Dc II)	5´primer: (918-942) = TGC CAA CAT TCA CCC GTT CTT CTT A 3´primer: (1335-1359) = CCT GTT TCC GGG TCG TAT GCT TTA T	56 °C x 34 0.2	442	M81190.1
Plakophilin (Plako) PKP1	5´primer: (1337-1361) = CCC GTG GAC CCC GAG GTC TTC TTC A 3´primer: (1580-1604) = CGG TGT AGG CGT TGC GGG CGT TGT A	64°C x 35 0.4	268	Z37975
Insulin-like growth factor-1 receptor (IGF1R)	5´primer: (186-212) = CAT CTC CAA CCT CCG GCC TTT TAC TCT 3´primer: (695-722) = CCC AGC CTG CTG CTA TTT CTT TTT CTA T	59°C x 37 0.3	538	X54980
IGF binding protein-2 (IGFBP2)	5´primer: (594-614) = TCC AGG CCG AGG TGA TGT TTG 3´primer: (394-414) = AGC GCC AGC CCC GAG CAG GTT	61°C x 33 0.2	221	NM_174555.1

Table 2-1. Continued.

Genes	Primer sequences and positions	Annealing temp (ºC) x cycle number and embryo equivalent	Fragment size (bp)	EMBL accession no.
IGF binding protein-3 (IGFBP3)	5´primer: (714-735) = AAC TTC TCC TCT GAG TCC AAG C 3´primer: (904-924) = CGT ACT TAT CCA CAC ACC AGC	56°C x 35 0.2	210	M76478
IGF binding protein-5 (IGFBP5)	5´primer: (403-423) = GGC AGT CGT GCG GCG TCT ACA 3´primer: (667-686) = CTT TCT GCG GTC CTT CTT CA	61°C x 35 0.2	284	XM_878464.1

Table 2-2. Effect of IGF-1 on cleavage rate, blastocyst development, cell number, cell allocation and apoptosis

Variable	Control	IGF-1
Cleavage rate d 3 (%)	85.3 ± 1.4%	81.9 ± 1.4%
Blastocysts/oocytes d 7 (%)	27.9 ± 1.3%	29.4 ± 1.3%
Advanced blastocysts/oocytes d 7 (%)	20.9 ± 1.0%	21.4 ± 1.0%
Total cell number	127.2 ± 2.8	124.7 ± 2.9
Number of inner cell mass cells	47.7 ± 1.4	44.0 ± 1.5†
Number of trophectoderm cells	83.0 ± 2.8	79.8 ± 3.1
Ratio trophectoderm:inner cell mass	2.1 ± 0.1	2.1 ± 0.1
Apoptotic blastomeres/total cells (%)	2.1 ± 0.3%	2.2 ± 0.3%

Data are least-squares means ± SEM. † $P < 0.06$.

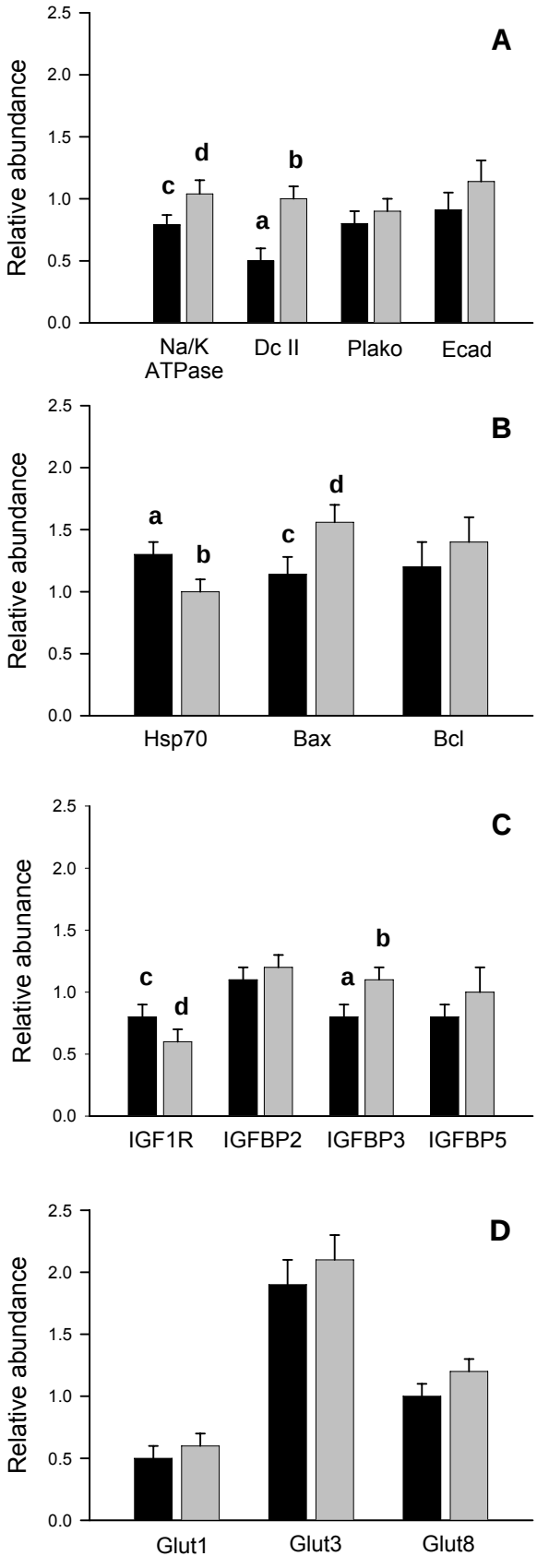
A
Relative abundance
2.5
2.0
1.5
1.0
0.5
0.0
c
d
a
b
Na/K ATPase
Dc II
Plako
Ecad
B
Relative abundance
a
b
c
d
Hsp70
Bax
Bcl
C
Relative abunance
c
d
a
b
IGF1R
IGFBP2
IGFBP3
IGFBP5
D
Relative abundance
Glut1
Glut3
Glut8

Figure 2-1. Effect of IGF-1 on the relative abundance of A) Na/K ATPase, Dc II, Plako, and E-cad, B) Hsp 70, Bax, and Bcl, C) IGF-1R, IGF-BP1, IGF-BP3, and IGF-BP5, and D) Glut1, Glut3, and Glut 8. Gray bars represent control embryos and black bars represent embryos treated with IGF-1 during culture. Data are least-squares means ± SEM. There were between 7 and 22 embryos per treatment. Bars for each transcript with different superscripts were statistically different (a:b $P < 0.05$) or tended to be statistically different (c:d $P < 0.08$).

CHAPTER 3
THE EFFECT OF IGF-1 SUPPLEMENTATION DURING IN VITRO BOVINE EMBRYO CULTURE ON SUBSEQUENT IN UTERO DEVELOPMENT TO DAY 14 OF GESTATION

Introduction

Early embryo development is coordinately regulated by several molecules secreted by the maternal reproductive tract, and in some cases, the embryo itself. Among such molecules, growth factors play an important role during preimplantation embryo development as they can regulate mitogenesis, differentiation, metabolism and apoptosis (Kane et al., 1997; Diaz-Cueto and Gerton, 2001; Hardy and Spanos, 2002). Despite their actions on early embryo development, growth factors are not routinely included in embryo culture medium. This may help to explain why embryos produced in vitro differ from their in vivo derived counterparts in terms of metabolism (Khurana and Niemann, 2000), gene expression (Bertolini et al., 2002a; Lazzari et al., 2002; Lonergan et al., 2003) and survival and development after transfer Farin and Farin, 1995; Hasler et al., 1995; Drost et al., 1999; van Wagtendonk de-Leeuw et al., 1998, 2000).

One growth factor that modifies embryonic physiology is insulin-like growth factor-1 (IGF-1). Addition of IGF-1 to culture medium can reduce the proportion of blastomeres that are apoptotic (Byrne et al., 2002b; Sirisathien and Brackett, 2003), alter the abundance of some developmentally important genes (Chapter 2), and increase cellular resistance to heat shock (Jousan and Hansen, 2004, 2006). Also, IGF-1 can increase development of bovine embryos to the blastocyst stage (Palma et al., 1997; Prelle et al., 2001; Byrne et al., 2002b; Makarevich and Markkula, 2002; Moreira et al., 2002b; Block et al., 2003; Sirisathien et al., 2003b; Chapter 4;) and increase blastocyst cell number (Byrne et al., 2002b; Moreira et al., 2002b; Sirisathien et al., 2003b), although these effects are not always observed (Prelle et al., 2001; Chapter 2)

Treatment of embryos with IGF-1 in culture can also improve pregnancy rates following transfer of embryos to heat-stressed, lactating dairy cows (Block et al., 2003; Chapter 4).

At present, the reasons for enhanced survival of IGF-1 treated embryos post-transfer are not clear. One possibility is that actions of IGF-1 on embryo development in vitro may allow for improved conceptus development or hormone secretion around the time of maternal recognition of pregnancy when the embryo undergoes elongation and secretes IFN-τ. Treatment of lactating cows with bovine somatotropin tended to increase the proportion of inseminated cows that had a recoverable conceptus at day 17 of pregnancy as well as conceptus size. In addition, the total amount of IFN-τ in uterine flushings was increased by somatotropin treatment (Bilby et al., 2006). Effects of somatotropin could be mediated by IGF-1, because concentrations in blood are elevated by somatotropin treatment. However, we cannot dismiss the possibility that these somatotropin-mediated actions are independent of IGF-1. The objective of the present study was to determine whether treatment of embryos with IGF-1 during culture would improve embryo survival to day 14 after ovulation. Moreover, it was hypothesized that embryos treated with IGF-1 would have increased length and interferon-τ secretion at day 14 compared to controls.

Materials and Methods

Materials

All materials were purchased from Sigma (St. Louis, MO) or Fisher Scientific (Fairlawn, NJ) unless specified otherwise. Sperm-Tyrode's Lactate, IVF-Tyrode's Lactate, and Hepes Tyrode's Lactate were purchased from Caisson Laboratories, Inc. (Logan, UT). These media were used to prepare Sperm-TALP, IVF-TALP, and Hepes-TALP as described previously (Parrish et al., 1986). Oocyte collection medium was TCM-199 with Hank's salts without phenol red (Atlanta Biologicals, Norcross, GA) and supplemented with 2% (v/v) bovine steer

serum (Pel-Freez, Rogers, AR), 2 U/mL heparin, 100 U/mL penicillin-G, 0.1 mg/mL streptomycin, and 1 mM glutamine. Oocyte maturation medium was TCM-199 (Invitrogen, Carlsbad, CA) with Earle's salts supplemented with 10% (v/v) bovine steer serum, 2 µg/mL estradiol 17-β, 20 µg/mL bovine FSH (Folltropin-V; Bioniche, Belleview, Ontario, Canada), 22 µg/mL sodium pyruvate, 50 µg/mL gentamicin sulfate, and 1 mM glutamine. Percoll was from Amersham Pharmacia Biotech (Uppsala, Sweden). Potassium simplex optimized medium that contained 1 mg/ml BSA was from Caisson. On the day of use, KSOM was modified to produce KSOM-BE2 as described previously [26]. Recombinant human IGF-1 was obtained from Upstate Biotech (Lake Placid, NY) and recombinant human IFN-α (3.84×10^8 IU/mg) was from EMD Biosciences (San Diego, CA). Prostaglandin $F_{2\alpha}$ was Lutalyse® from Pharmacia & UpJohn (New York, NY) and GnRH was Cystorelin® from Merial (Duluth, GA). Controlled internal drug releasing devices were purchased from Pfizer (New York, NY) and lidocaine was from Pro Labs (St. Joseph, MO)

In Vitro Embryo Production

Embryos were produced in vitro as described previously (Soto et al., 2003). Briefly, COCs were obtained by slicing 2- to 10-mm follicles on the surface of ovaries (predominantly beef cattle) obtained from Central Beef Packing Co. (Center Hill, FL). Those COCs with multiple layers of compact cumulus cells were washed two times in OCM and used for subsequent steps. Groups of 10 COCs were placed in 50-µl drops of OMM overlaid with mineral oil and matured for 21-24 h at 38.5°C in an atmosphere of 5% (v/v) CO_2 in humidified air. Matured COCs were then washed once in HEPES-TALP and transferred in groups of 30 to 4-well plates containing 600 µl of IVF-TALP and 25 µl of PHE (0.5 mM penicillamine, 0.25 mM hypotaurine, and 25 µM epinephrine in 0.9% [w/v] NaCl) per well and fertilized with ~1×10^6 Percoll-purified spermatozoa from a pool of frozen-thawed semen from three bulls. Depending

on the experiment, COCs and spermatozoa were allowed to co-incubate for 20-24 h at 38.5°C in an atmosphere of 5% (v/v) CO_2 in humidified air. After fertilization, putative zygotes were removed from fertilization wells, denuded of cumulus cells by vortex mixing in HEPES-TALP containing 1000 U/ml hyaluronidase, and randomly placed in groups of 25 in 50-µl drops of either KSOM-BE2 or KSOM-BE2 containing 100 ng/mL as described previously [21]. All drops of embryos were overlaid with mineral oil and cultured at 38.5°C in an atmosphere of 5% CO_2 (experiment 1) or 5% CO_2, 5% O_2 and 90% N_2 (experiment 2). The proportion of cleaved oocytes was recorded on day 3 after insemination and the proportion of blastocysts and advanced blastocysts (expanded and hatched) was recorded on day 7 (experiment 2) or day 8 (experiment 1).

Experiment 1 (Group Transfer of Embryos)

Animals

Non-lactating Holstein cows at the University of Florida Dairy Research Unit (Hague, FL; 29.77904 N, 82.48001 W) were used as embryo transfer recipients. Cows were kept on pasture and supplemented with corn silage, grass hay and free-choice mineral. Animals were synchronized for timed embryo transfer using a modified Ovsych protocol with the inclusion of a CIDR (El-Zarhkouny et al., 2004). Cows received 100 µg of GnRH (i.m.) and a CIDR (intravaginal deposition) on day -10. On day -3, cows received 25 mg PGF and the CIDR was removed. A second injection of GnRH was administered on day 0 (day of anticipated ovulation). Also on day 0, the ovaries of all cows were scanned using an Aloka 500 ultrasound (Aloka America, Wallingford, CT) equipped with a 5 MHz linear array transducer to determine the presence or absence of a preovulatory follicle.

Embryo transfer

On day 8 after fertilization, grade 1 blastocyst and expanded blastocyst stage embryos (Robertson and Nelson, 1998) were harvested from culture. Selected embryos were placed into holding medium [Hepes-TALP containing 10% (v/v) fetal bovine serum and 100 μM β-mercaptoethanol] and loaded into 0.25 cc French straws in groups of 7-12 (depending on the replicate). Embryos were loaded so that similar numbers of blastocyst and expanded blastocyst stage embryos were placed into each straw across both treatment groups. Once embryos were loaded, the straws were then placed into a portable incubator set at 39º C and transported to the farm for transfer to recipients.

At day 7 after anticipated ovulation, the ovaries of all cows were scanned again using ultrasonography to determine the presence or absence of a corpus luteum. Cows were selected for transfer based on 2 criteria: 1) cows that did not have a preovulatory follicle or a corpus luteum on day 0 but that did have a corpus luteum on day 7 (i.e., cows that ovulated after the PGF injection but before the 2nd GnRH injection of OvSynch) or 2) cows that had a preovulatory follicle on day 0 that was replaced by the presence of a corpus luteum on day 7. A total of 62 cows were selected as embryo transfer recipients based on these criteria. Selected recipients received an epidural block of 5 mL lidocaine (2%) and groups of embryos were then randomly transferred to the uterine horn ipsilateral to the ovary with a corpus luteum.

Embryo recovery, evaluation and culture

On day 14 after anticipated ovulation, both the ipsilateral and contralateral uterine horns of each recipient were flushed with Dulbecco's Phosphate Buffered Saline (DPBS) to recover embryos. For 3 replicates, recipients were slaughtered, the reproductive tracts were collected and the uterine horns were flushed with 100 mL of DPS. For 7 replicates, embryos were recovered using non-surgical embryo recovery techniques. The uterine horns of each recipient

were flushed separately using 18-20 French Foley catheters and the flushing procedure continued until 500 mL of DPBS had been recovered from each uterine horn.

Following embryo recovery, embryo length, embryo stage, and the presence or absence of an embryonic disc was assessed by light microscopy using a stereomicroscope. The stage of each embryo was classified into one of 4 groups based on embryo shape: 1) spherical, 2) ovoid, 3) tubular and 4) filamentous. After all measurements were recorded, embryos were then placed into 5 mL of TCM-199 containing 200 U/mL penicillin-G and 0.2 mg/mL steptomycin and cultured at 38.5º C in 5% CO_2. After approximately 24 hrs of culture, the medium was harvested and stored at -80 º C until further processing.

Experiment 2 (Single-Embryo Transfer)

Animals

For 7 replicates, non-lactating Holstein cows at the University of Florida Dairy Research Unit (Hague, FL; 29.77904 N, 82.48001 W) were used for embryo transfer recipients as described for experiment 1. For 4 replicates, lactating Holstein cows at a commercial dairy in Florida (Bell, FL; 29.75578 N, 82.86188 W) were used as embryo transfer recipients. Lactating cows between 64 and 615 days in milk (mean = 193) were housed in a free-stall barn, fed a total-mixed ration and milked 3 times per day. Regardless of location, animals were synchronized for timed embryo transfer using the Ovsych protocol (Pursley et al., 1997). Cows received 100 µg of GnRH (i.m.) on day -10 followed 7 days later (day - 3) by 25 mg PGF. On day -1, a second injection of GnRH was administered and the ovaries of all cows were scanned as described in experiment 1 to determine the presence or absence of a preovulatory follicle. Day 0 was defined as the day of anticipated ovulation.

Embryo transfer

On day 7 after fertilization, grade 1 blastocyst and expanded blastocyst stage embryos (Robertson and Nelson, 1998) were harvested from culture. Selected embryos were placed into 1.5 mL of holding medium in 2 mL microcentrifuge tubes, placed into a portable incubator set at 39ºC and transported to the farm for transfer to recipients. Upon arrival at the farm, grade 1 embryos were loaded individually into 0.25 cc French straws in holding medium.

At day 7 after anticipated ovulation, the ovaries of all cows were scanned again using ultrasonography to determine the presence or absence of a corpus luteum. Cows were selected for transfer based on the criteria described in experiment 1. A total of 56 non-lactating and 35 lactating cows were selected as embryo transfer recipients. Selected recipients received an epidural block of 5 mL of lidocaine (2%) and a single embryo was then randomly transferred to the uterine horn ipsilateral to the ovary with a corpus luteum.

Embryo recovery, evaluation and culture

Non-surgical embryo recovery procedures were used on day 14 after anticipated ovulation as described in experiment 1. Embryos were also evaluated and cultured as in experiment 1 except that the presence or absence of an embryonic disc was not recorded in experiment 2.

Analysis of Interferon-τ Secretion

The quantity of biologically active IFN-τ in embryo culture medium after 24 h culture was determined using an antiviral assay based on the inhibition of vesicular stomatitis virus-induced lysis of Madin-Darby bovine kidney cells (Micheal et al., 2006). The dilution of sample that prevented virus-induced lysis by 50% was converted to ng/mL of IFN-τ by comparison to activity of a recombinant bovine IFN-τ standard (Ealy et al., 2001) that was included in the assay. The specific activity of the bovine IFN-τ standard (1.68×10^8 IU/mg) was determined by

comparison to a recombinant human IFN-α standard also included in the assay (EMD Biosciences, San Diego, CA; 3.84 x 10^8 IU/mg).

Statistical Analysis

Percentage data were transformed by arcsin transformation before analysis. Probability values for percentage data are based on analysis of arcsin-transformed data while least-squares means are from analysis of untransformed data.

The proportion of oocytes that cleaved, that developed to the blastocyst stage on day 7 (experiment 2) or day 8 (experiment 1) and that developed to advanced blastocyst stages (expanded, hatching or hatched) on day 7 (experiment 2) or day 8 (experiment 1) were calculated for each replicate in each experiment. Treatment effects were analyzed using least-squares analysis of variance using the General Linear Models procedure of SAS (SAS for Windows, version 9.0, SAS Inst., Inc., Cary, NC). The model included the main effects of replicate and treatment. All values reported are least-squares means ± SEM.

Recovery rate in experiment 1, as well as embryo length and IFN-τ secretion in both experiments, were analyzed by analysis of variance using the GLM procedure of SAS. The statistical model in experiment 1 included treatment, flush type (i.e. slaughter vs. live animal), cow(flush type x treatment) and treatment x flush type. For experiment 2, the statistical model included replicate, treatment, lactation and all two-way interactions. For IFN-τ secretion, data were analyzed with and without embryo length as a covariate. All values obtained from the GLM procedure are reported as least-squares means ± SEM. The correlation between embryo length and IFN-τ secretion was analyzed using the CORR procedure of SAS.

Embryo recovery in experiment 2 and the proportion of embryos that had a visible embryonic disc at day 14 after ovulation in experiment 1 were analyzed by logistic regression

using the LOGISTIC procedure of SAS. The statistical model for each experiment was the same as described above. The data are reported as the actual percentage.

Embryo stage in both experiments was analyzed using both the LOGISTIC and CATMOD procedures of SAS. The statistical models for each experiment were the same as described above. The statistical values obtained after analysis with LOGISTIC and CATMOD were similar and only statistical inferences from the LOGISTIC analysis are reported. Data are reported as the actual percentage.

Results

Experiment 1(Group Transfer of Embryos)

Embryo development in vitro

Addition of IGF-1 to culture increased ($P < 0.05$) cleavage rate on day 3 (Control – 80.9 ± 0.8% vs. IGF-1 – 84.0 ± 0.8%). However, there was no effect of IGF-1 on the proportion of oocytes that developed to the blastocyst stage (Control – 27.3 ± 1.6% vs. IGF-1 – 28.7 ± 1.6%) or advanced blastocyst stages (Control – 14.9 ± 0.6% vs. IGF-1 – 14.7 ± 0.6%) on day 8 after insemination.

Embryo recovery and development at day 14

Supplementation of culture medium with IGF-1 did not affect the proportion of embryos recovered at day 14 (Table 3-1). Among embryos recovered, there was also no effect of IGF-1 on embryo length, IFN-τ secretion, or the proportion of embryos with a visible embryonic disc (Table 3-1). In addition, treatment with IGF-1 did not affect embryo stage at day 14 (Table 3-2).

Recovery rate and embryo length were affected by flush type (i.e., slaughter vs. live animal). Recovery rate tended to be greater ($P < 0.06$) for embryos that were recovered after slaughter than for embryos collected by non-surgical procedures (37.8 ± 6.2% vs. 21.8 ± 4.1%, respectively). In addition, embryo length was greater ($P < 0.01$) for embryos recovered

following slaughter than for embryos recovered from live recipients (7.7 ± 0.87 mm vs. 3.7 ± 0.85 mm, respectively). There was no effect of flush type on IFN-τ secretion, the proportion of embryos with a visible embryonic disc or embryo stage at day 14. Moreover, there was no interaction between embryo treatment and flush type on any of the variables analyzed.

Embryo length was a significant covariate for IFN-τ secretion ($P < 0.001$) and there was a positive correlation (r = 0.5; $P < 0.001$) between embryo length and IFN-τ secretion (Figure 3-1).

Experiment 2 (Single Embryo Transfer)

Embryo development in vitro

Addition of IGF-1 to embryo culture did not affect cleavage rate on day 3 (control – 85.2 ± 1.2% vs. IGF-1 – 85.3 ± 1.2%) or the proportion of oocytes that developed to advanced blastocyst stages on Day 7 (control – 11.3 ± 2.2% vs. IGF-1 – 14.2 ± 2.2%). However, the proportion of oocytes that developed to the blastocyst stage on day 7 tended to be increased ($P < 0.08$) for IGF-1 treated embryos compared to controls (23.8 ± 1.8% vs. 18.6 ± 1.8%, respectively).

Embryo recovery and development at day 14

There was a tendency ($P = 0.10$) for a greater recovery rate at day 14 for recipients that received IGF-1 treated embryos compared to control embryos (Table 3-3). However, there was no effect of IGF-1 on embryo length, IFN-τ secretion (Table 3-3) or embryo stage (Table 3-4) for recovered embryos.

There was no effect of lactation or an interaction between embryo treatment and lactation on recovery rate, embryo length, IFN-τ secretion, or embryo stage at day 14 after ovulation.

As in experiment 1, embryo length was a significant covariate for IFN-τ secretion ($P < 0.001$) and there was a strong positive correlation ($r = 0.9$; $P < 0.001$) between embryo length and IFN-τ secretion (Figure 3-2).

Discussion

Insulin-like growth factor-1 promotes post-transfer embryonic survival in heat-stressed lactating recipients (Block et al., 2003; Chapter 4). This effect was observed as early as day 21 of pregnancy, because more cows receiving IGF-1 treated embryos had elevated progesterone concentrations at Day 21 (Chapter 4). Such a result suggests that IGF-1 could affect embryonic survival to the time of maternal recognition of pregnancy and enhance the ability of the day 14 embryo to block luteolysis through increased IFN-τ secretion. The current results indicate that IGF-1 may increase embryo survival at day 14 after ovulation but there was no evidence that it affected the IFN-τ signaling capacity of the embryo. The increased survival of IGF-1 treated embryos was only observed when single embryo transfers were performed and this result is interpreted to indicate that the use of group embryo transfer can obscure some effects of culture conditions on embryo survival.

Group embryo transfer has been used previously to test the effects of culture conditions on post-transfer embryo survival (Rexroad and Powell, 1999; Lazzari et al., 2002; Fischer-Brown et al., 2005). However, the results of the present study suggest that positive effects of embryo culture treatments may be masked by the transfer of multiple embryos. Although IGF-1 treatment tended to increase embryo survival in experiment 2 when each recipient received a single embryo, there was no effect of IGF-1 on embryo survival in experiment 1 when groups of 7-12 embryos were transferred to each recipient. One possibility is that IGF-1 treated embryos secrete, or induce the uterus to secrete, factors that allow for improved embryo survival. When

multiple embryos are present within a single uterine horn, the amounts of embryotrophic molecules secreted by individual embryos may be less determinative of embryonic survival than when individual embryos are present. Cooperation between embryos has been observed in vitro where embryo development is improved when embryos are cultured in groups rather than individually (Paria and Dey, 1990; Lane and Gardner, 1992). Also, competition for uterine factors induced by IGF-1 treated embryos may dampen the effect of IGF-1 on embryo survival. Finally, it is possible that a positive effect of IGF-1 on embryo survival in experiment 1 may have been blocked by limited uterine capacity. While the effect of uterine capacity on early embryo survival in cattle is not well characterized, differences between embryos recovered in experiment 1 and 2 suggest that uterine crowding may have affected embryo development. Both embryo length and IFN-τ secretion were lower for embryos recovered in experiment 1 than in experiment 2 (Table 3-1 vs. Table 3-3) and a greater percentage of embryos recovered at day 14 in experiment 1 were retarded in development compared to embryos recovered in experiment 2 (Table 3-2 vs. Table 3-4).

The recovery of viable embryos represents one important aspect of embryo survival to day 14. While IGF-1 tended to increase embryo recovery rates at day 14 in experiment 2, the embryos that do survive do not appear superior in terms of capacity for communication with the maternal environment because there was no effect of IGF-1 on embryo length or stage of development, or IFN-τ secretion. Although not statistically significant, there was a numeric shift in the distribution of embryos within each stage category at day 14. Specifically, a greater percentage of control embryos were at the tubular and filamentous stage of development (90.1% vs. 75% for control and IGF-1, respectively) while more IGF-1 treated embryos were at the spherical and ovoid stage (8.3% vs. 25.1% for control and IGF-1, respectively). The improved

embryo survival observed in experiment 2 may be related to other cellular differences between IGF-1 and control embryos. Recently, it was reported that addition of IGF-1 to embryo culture altered the relative abundance of some developmentally important gene transcripts (Chapter 2). Embryos treated with IGF-1 had an increased abundance of Na/K and desmocollin II transcripts which are critical for the mechanical integrity of the embryo (Fleming et al., 1991; Collins et al., 1995; Watson and Barcraft, 2001; Violette et al., 2006). In addition, IGF-1 treatment decreased the abundance of Hsp70 mRNA transcripts which are generally increased following culture in vitro (Lazzari et al., 2002; Sagirkaya et al., 2006). Such differences may be important for embryo survival to day 14 of gestation.

Treatment of embryos with IGF-1 tended to enhance survival at day 14 in experiment 2 even though transfers were conducted during the cool season. This finding is contradictory to a recent report in which the transfer of IGF-1 treated embryos to lactating dairy cows increased pregnancy rates at day 21 of gestation, but only under heat stress conditions (Chapter 4). It is not clear why such a discrepancy would occur. In the absence of heat stress, perhaps, IGF-1 treated embryos experience greater losses after day 14. Alternatively, the effect of IGF-1 treatment of embryos depends upon the physiological status of the recipient in ways that extend beyond meteorological factors and recipients used here were responsive to treatment.

Treatment of lactating dairy cows with bST at the time of insemination and 11 days later tended to increase the proportion of recipients that had a recoverable conceptus at day 17 (Bilby et al.,2006). Moreover, bST increased conceptus length and total IFN-τ in uterine flushings. The effects of bST could be mediated by either bST or IGF-1 because concentrations of both are increased by bST treatment (Bilby et al.,2006). In experiment 2, treatment of embryos with IGF-1 from Day 1-7 after insemination tended to increase the proportion of recipients that had a

recoverable conceptus on day 14. However, IGF-1 had no affect on conceptus length or IFN-τ secretion. These results suggest that IGF-1 may help mediate the actions of bST on embryo survival. However, bST effects on conceptus length and IFN-τ secretion may not be mediated by IGF-1 or alternatlively, actions of IGF-1 on conceptus length and IFN-τ secretion don't occur until after day 7.

Embryo recovery rates at day 14 in experiment 1 and experiment 2 were 27.6% (153/554) and 33.7% (28/83), respectively. Similar embryo recovery rates have been reported previously for in vitro produced embryos recovered at Day 14 (Rexroad and Powell, 1999; Fischer- Brown et al., 2005). There was however, an effect of flush type on recovery rate and embryo length in experiment 1. In particular, more embryos were recovered and embryos were longer when recipients were flushed following slaughter compared to non-surgical embryo recovery using live recipients. While the mean embryo length for embryos recovered using non-surgical recovery procedures (3.7 ± 0.85 mm) was similar to previous reports in which embryos were recovered from live recipients at Day 14 following group embryo transfer [1.3-4.9 mm;32,33,42], the mean embryo length for embryos recovered after slaughter was much longer (7.7 ± 0.87 mm). These results suggest the possibility that non-surgical embryo recovery is not the optimal method for recovering intact, elongated embryos following group embryo transfer.

Culture conditions have a significant effect on the proportion of embryos at day 14 with a visible embryonic disc (Fischer-Brown et al., 2005). Embryos without a visible disc are not capable of establishment of pregnancy following transfer (Fischer-Brown et al., 2005). There was however, no effect of IGF-1 on the proportion of embryos recovered at day 14 in experiment 1 that had a visible embryonic disc. Although detection of the embryonic disc using stereomicroscopy can be imprecise, the proportion of embryos with a visible embryonic disc in

experiment 1 (88/114 = 77.2%) is similar to previous reports (Rexroad and Powell, 1999; Fischer-Brown et al., 2005).

Addition of IGF-1 to embryo culture improves embryo development in several studies (Palma et al., 1997; Prelle et al., 2001; Byrne et al., 2002b; Makarevich and Markkula, 2002; Moreira et al., 2002b; Block et al., 2003; Sirisathien et al., 2003b; Chapter 4), but not in some cases (Herrler et al., 1992; Palma et al., 1997; Chapter 2). In the present set of experiments, IGF-1 treatment tended to increase the proportion of oocytes that developed to the blastocyst stage at day 7 in experiment 2, but there was no effect of IGF-1 on blastocyst development at day 8 in experiment 1. Similar results were also observed in a recent study from our laboratory in which IGF-1 increased embryo development to the blastocyst stage on Day 7, but there was no effect on day 8 (Chapter 4). Inconsistencies in the effect of IGF-1 on embryo development may be partly explained by differences in culture systems since there are reports that the ability of IGF-1 to stimulate embryonic development depend upon culture conditions (Herrler et al., 1992; Palma et al., 1997).

Table 3-1. Effect of IGF-1 on recovery rate, embryo length, IFN-τ secretion and embryonic disc formation at Day 14 after ovulation in experiment 1.

Variable	Control	n	IGF-1	n
Recovery Rate (%)	30.4 ± 5.1	294	29.2 ± 5.5	260
Embryo Length (mm)	5.4 ± 5.5	83	5.9 ± 5.8	70
IFN-τ (ng/mL)	29.2 ± 7.5	51	22.5 ± 7.5	54[a]
	26.8 ± 9.0	51	22.5 ± 8.5	54[b]
Embryonic Disc (%)	75.8	62	77.9	54

[a] Analysis includes embryo length as a covariate. [b] Analysis performed without embryo length as a covariate.

Table 3-2. Effect of IGF-1 on embryo stage at Day 14 after ovulation in experiment 1

Stage (%)	Control	n	IGF-1	n
Spherical	13.3	11	12.9	9
Ovoid	32.5	27	32.9	23
Tubular	33.7	28	32.9	23
Filamentous	20.5	17	21.4	15

Table 3-3. Effect of IGF-1 on recovery rate, embryo length and IFN-τ secretion at Day 14 after ovulation in experiment 2

Variable	Control	N	IGF-1	n
Recovery Rate (%)	26.1	46	43.2[a]	37
Embryo Length (mm)	24.1 ± 9.2	12	28.8 ± 8.6	15
IFN-τ (ng/mL)	284.5 ± 56.2	10	329.2 ± 47.5[b]	14
	264.0 ± 131.1	10	354.5 ± 113.6[c]	14

[a] Treatment $P = 0.1$. [b] Analysis includes embryo length as a covariate. [c] Analysis performed without embryo length as a covariate.

Table 3-4. Effect of IGF-1 on embryo stage at Day 14 after ovulation in experiment 2.

Stage (%)	Control	n	IGF-1	n
Spherical	0	0	6.3	1
Ovoid	8.3	1	18.8	3
Tubular	33.3	4	37.5	6
Filamentous	58.3	7	37.5	6

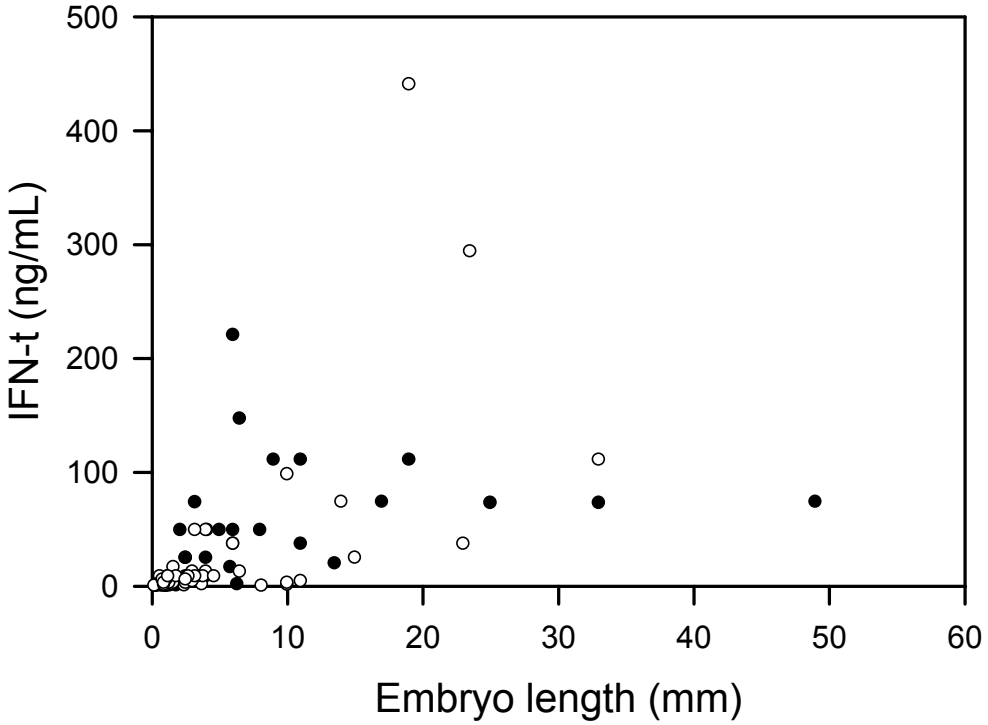

Figure 3-1. Relationship between embryo length and IFN-τ secretion for control embryos (black circles) and IGF-1 embryos (open circles) recovered at Day 14 in experiment 1. The correlation between embryo length and IFN-τ secretion was r = 0.5 ($P < 0.001$)

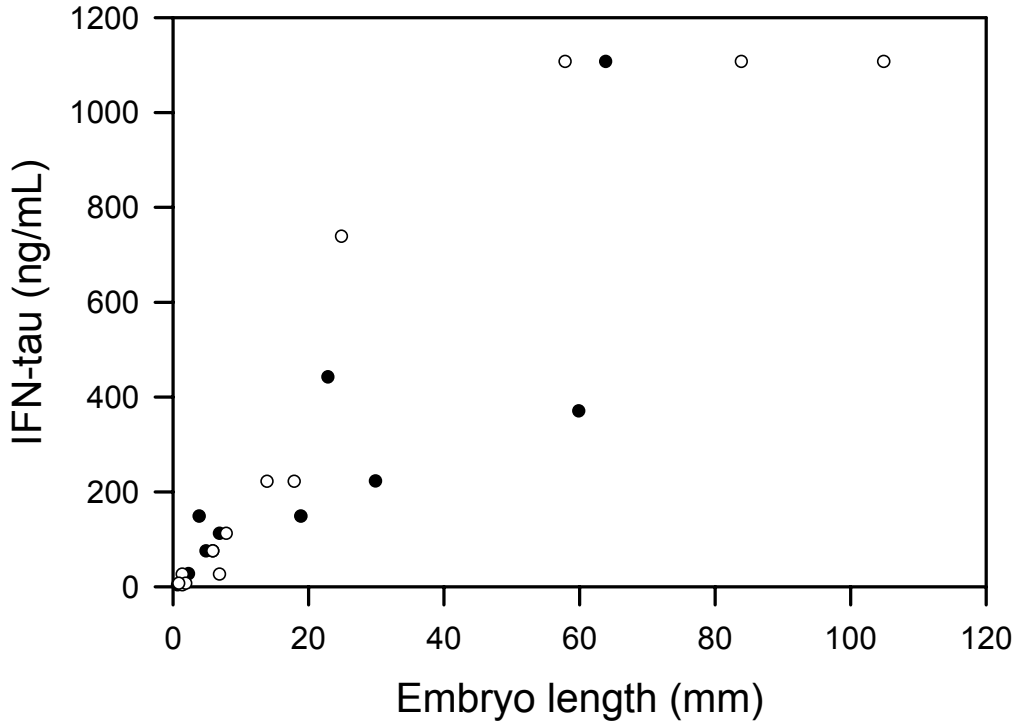

Figure 3-2. Relationship between embryo length and IFN-τ secretion for control embryos (black circles) and IGF-1 embryos (open circles) recovered at Day 14 in experiment 2. The correlation between embryo length and IFN-τ secretion was r = 0.9 ($P < 0.001$).

CHAPTER 4
INTERACTION BETWEEN SEASON AND CULTURE WITH INSULIN-LIKE GROWTH FACTOR-1 ON SURVIVAL OF IN-VITRO PRODUCED EMBRYOS FOLLOWING TRANSFER TO LACTATING DAIRY COWS

Introduction

Exposure to heat stress reduces fertility in lactating dairy cows (Badinga et al., 1985; Lopez-Gatius, 2003). While early embryonic development is very sensitive to the deleterious effects of heat stress, embryos become more resistant as development progresses (Ealy et al., 1993; Edwards and Hansen, 1997). Thus, embryo transfer can be used to bypass the period during which the embryo is most sensitive to heat stress and improve fertility as compared to artificial insemination (Putney et al., 1989; Ambrose et al., 1999; Drost et al., 1999; Al-Katanani et al., 2002). There does remain, however, some detrimental effects of heat stress on pregnancy rates in embryo transfer recipients (Vasconcelos et al., 2006; Galvao et al., 2006).

One strategy to increase pregnancy success for transfer of in vitro produced embryos is to alter embryo culture conditions to improve post-transfer viability of embryos. Addition of IGF-1 to culture medium can increase development of bovine embryos to the blastocyst stage (Palma et al., 1997; Prelle et al., 2001; Byrne et al., 2002b; Moreira et al., 2002b; Block et al., 2003; Sirisathien et al., 2003b), increase blastocyst cell number (Byrne et al., 2002b; Moreira et al., 2002b; Sirisathien et al., 2003b) and reduce the proportion of blastomeres that are apoptotic (Byrne et al., 2002b; Sirisathien and Brackett, 2003). Treatment of bovine preimplantation embryos with IGF-1 also improves resistance to heat shock by reducing effects of elevated temperature on blastomere apoptosis and development to the blastocyst stage (Jousan and Hansen, 2004, 2006).

Recently, it was demonstrated that lactating recipient dairy cows exposed to heat stress had higher pregnancy rates when receiving an embryo treated with IGF-1 during culture as compared to control embryos (Block et al., 2003). It is not clear whether this beneficial effect of IGF-I on post-transfer survival is due to actions of IGF-1 on embryonic development in general or, alternatively, is related to the thermoprotective actions of IGF-1 on bovine embryo development (Jousan and Hansen, 2004, 2006). Therefore, the objective of the present study was to determine whether the beneficial effect of culturing embryos in the presence of IGF-1 on post-transfer survival would be apparent regardless of season or under heat stress conditions only.

Materials and Methods

Materials

All materials were purchased from Sigma (St. Louis, MO) or Fisher Scientific (Fairlawn, NJ) unless specified otherwise. Sperm-Tyrode's Lactate, IVF-Tyrode's Lactate, and Hepes Tyrode's Lactate were purchased from Caisson Laboratories, Inc. (Logan, UT). These media were used to prepare Sperm-TALP, IVF-TALP, and Hepes-TALP as described previously (Parrish et al., 1986). Oocyte collection medium was TCM-199 with Hank's salts without phenol red (Atlanta Biologicals, Norcross, GA) and supplemented with 2% (v/v) bovine steer serum (Pel-Freez, Rogers, AR), 2 U/mL heparin, 100 U/mL penicillin-G, 0.1 mg/mL streptomycin, and 1 mM glutamine. Oocyte maturation medium was TCM-199 (Invitrogen, Carlsbad, CA) with Earle's salts supplemented with 10% (v/v) bovine steer serum, 2 μg/mL estradiol 17-β, 20 μg/mL bovine FSH (Folltropin-V; Bioniche, Belleview, Ontario, Canada), 22 μg/mL sodium pyruvate, 50 μg/mL gentamicin sulfate, and 1 mM glutamine. Percoll was from Amersham Pharmacia Biotech (Uppsala, Sweden). Potassium simplex optimized

medium that contained 1 mg/ml BSA was from Caisson. On the day of use, KSOM was modified to produce KSOM-BE2 as described previously (Block et al.,2003). Recombinant human IGF-1 was obtained from Upstate Biotech (Lake Placid, NY). Prostaglandin $F_{2\alpha}$ was Lutalyse® from Pharmacia & UpJohn (New York, NY) and GnRH was Cystorelin® from Merial (Duluth, GA). Controlled internal drug releasing devices were purchased from Pfizer (New York, NY) and lidocaine was from Pro Labs (St. Joseph, MO)

Animals

The experiment was conducted between March 2005 and September 2006 at four locations: Farm 1 (Live Oak, Florida; 30.29434 N, 82.98607 W), Farm 2 (Hague, Florida; 29.77904 N, 82.48001 W), Farm 3 (Bell, Florida; 29.75578 N, 82.86188 W), and Farm 4 (Okeechobee, Florida; 27.24126 N, 80.82988 W). The maximum daily temperatures and average relative humidities for March 15, 2005 through February 9, 2006 (from 10 days before transfers were initiated until completion of all pregnancy diagnoses) are shown in Figure 4-1 for data from nearby weather stations at Live Oak, Florida (Farm 1), Alachua, Florida (Farms 2 and 3), and Ft. Pierce, Florida (Farm 4) as recorded by the Florida Automated Weather Network (http://fawn.ifas.ufl.edu/).

At Farm 1, 53 primiparous and multiparous lactating Holstein x Jersey cows between 63 and 807 DIM (mean = 184.3) were used as recipients from March through April, 2005. Cows were housed outdoors on a dirt lot with access to shade cloth structures and sprinklers. All recipients were fed a total mixed ration (TMR) and milked 3 times per day. Overall, 5 replicates were completed with between 7 and 15 recipients per replicate. At Farm 2, a total of 99 primiparous and multiparous lactating Holstein cows between 87 and 1,014 days in milk (DIM; mean = 317.1) were used as recipients

from March through September, 2005. All recipients were housed in a free stall barn equipped with fans and sprinklers, fed a TMR and milked 3 times per day. A total of 96 recipients received bovine somatotropin as per manufacturer's instructions (Monsanto, Chesterfield, MO). Overall, 6 replicates were completed with between 11 and 28 recipients per replicate. At farm 3, a total of 114 primiparous and multiparous lactating Holstein cows between 36 and 789 DIM (mean = 222.9) were used as recipients from July 2005 through January 2006. All recipients were housed in a free stall barn equipped with fans and sprinklers, fed a TMR and milked 3 times per day. A total of 82 recipients received bST as per manufacturer's instructions. Overall, 7 replicates were completed with between 10 and 20 recipients per replicate. At Farm 4, a total of 44 primiparous and multiparous lactating Holstein cows between 68 and 84 DIM (mean = 78.5) were used as recipients during November 2005. All recipients were housed in a free stall barn equipped with fans and sprinklers, fed a TMR and milked 3 times per day. A total of two recipients received bST as per manufacturer's instructions. Overall, 4 replicates were completed with between 8 and 13 recipients per replicate.

Cows at all four farms were synchronized for timed embryo transfer. Regardless of the protocol used, day 0 was defined as the day of anticipated ovulation. Cows at Farm 1 were synchronized using a modified OvSynch protocol (El-Zarkouny et al., 2004). Cows received 100 μg of GnRH (i.m.) and a CIDR (intravaginal deposition) on day -10. On day -3, cows received 25 mg PGF and the CIDR was removed. A second injection of GnRH was administered on day -1. Cows at Farm 2 and Farm 3 were synchronized as described for Farm 1 without the inclusion of a CIDR (Pursley et al.,

1997). For Farm 4, cows were synchronized using two injections of PGF 14 days apart (day-18 and day-4).

Cows at all locations were palpated at Day 7 after anticipated ovulation using an Aloka 500 ultrasound equipped with a 5 MHz linear array transducer to diagnose the presence of a corpus luteum. All cows having a corpus luteum received an epidural block of 5 mL of lidocaine (2%) and a single embryo was then transferred to the uterine horn ipsilateral to the ovary with a corpus luteum.

Pregnancy Diagnosis and Calving Data

Pregnancy at day 21 after ovulation was assessed by measurement of peripheral blood progesterone concentrations. Blood samples were taken on day 21 after anticipated ovulation by coccygeal venipuncture into evacuated heparinized tubes (Becton Dickinson, Franklin Lakes, NJ). Following collection, blood samples were placed in an ice chest until further processing at the laboratory (approximately 3 to 8 h). Blood samples were centrifuged at 3,000 x *g* for 15 min at 4 C. Plasma was separated and stored at -20 C until assayed for progesterone. Plasma progesterone concentrations were determined using the Coat-a-Count® progesterone RIA kit (Diagnostic Products Corp., Los Angeles, CA). The sensitivity of the assay was 0.1 ng/mL and the intrassay CV was 5.6%. Cows were classified as pregnant if the progesterone concentration was $\geq$ 2.0 ng/ml. Pregnancy was also diagnosed at ~day 30 of gestation (range = day 27-32) using ultrasonography and again at ~day 45 of gestation (range = day 41-49 by rectal palpation per rectum. Calving data was recorded for Farms 1, 2, and 4. Data included calf sex and gestation length (Farms 1, 2 and 4) and calf birth weight (Farm 2). In addition, the calf birth weights and calf sexes of cows (n=54) that were bred by artificial insemination during the week prior to each embryo transfer replicate at Farm 2 were also recorded.

Embryo Production

For Farms 1, 2, and 4, Holstein COCs were purchased from BOMED, Inc (Madison, WI; n = 3 replicates), Trans Ova Genetics (Sioux Center, IA; n = 3 replicates), or Evergen Biotechnologies (Storrs, CT; n = 9 replicates). Following collection, COCs were placed into 2 mL cryovials (approximately 50-115 COCs/cryovial) containing maturation medium and shipped overnight in a portable incubator set at 38.5°C to the laboratory in Gainesville, FL. For Farm 3 (n=7 replicatges), COCs were collected as described previously [23] from ovaries (predominately beef cattle) obtained from Central Packing Co. (Center Hill, FL). Regardless of farm, all COC's were allowed to mature for 21-24 hrs.

In vitro fertilization and embryo culture were conducted as described elsewhere (Soto et al., 2003) and all procedures were similar for each farm unless noted otherwise. Following maturation, COCs were washed once in Hepes-TALP and then fertilized with frozen-thawed semen. For farms 1 and 2, a single Holstein bull was used for each farm. For farm 3, semen from three randomly selected bulls was used and three different bulls were used for each replicate. For farm 4, two Holstein bulls were used and alternated for each replicate. Following 20-24 hrs of coincubation, presumptive zygotes were then cultured in KSOM-BE2 with or without 100 ng/mL IGF-1 as described elsewhere (Block et al., 2003). For the first 4 replicates (Farm 1 and 2), presumptive zygotes were cultured in a humidified atmosphere of 5% CO_2 and for the remaining 18 replicates (Farms 1-4) presumptive zygotes were cultured in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2. Cleavage rate was recorded on day 3 and the proportion of oocytes developing to the blastocyst stage was recorded on day 8 (first seven replicates; Farms 1 and 2) and 7 (n = 15 replicates; Farms 2-4).

Grade 1 morula, blastocyst, and expanded blastocyst stage embryos (Robertson and Nelson, 1998) were harvested on day 7 (n = 15 replicates) or day 8 (n = 7 replicates) after insemination and transported to the farm using one of two different methods. For the first 6 replicates, harvested embryos were loaded into 0.25 cc French straws in holding medium (Hepes-TALP containing 10% fetal bovine serum and 100 μM β-mercaptoethanol). Straws containing selected embryos were then placed horizontally into a portable incubator (Minitube, Verona ,WI) at 39°C and transported to the respective farm. For the remaining 16 replicates, harvested embryos were placed into 2 mL microcentrifuge tubes containing holding medium, placed into a portable incubator at 39°C and transported to the respective farm. Once at the farm, embryos were then loaded into 0.25 French straws in holding medium. Regardless of transportation method, straws containing embryos were loaded into a 21-inch transfer pipette (IMV Technologies, L'Aigle, France) and randomly transferred to recipients. Of the harvested embryos, 79 were blastocysts and 232 were expanded blastocysts.

Statistical Analysis

The proportion of oocytes that cleaved, that developed to the blastocyst stage on the day of blastocyst harvest (i.e. day 7 or 8 after insemination), and the proportion that developed to advanced blastocyst stages (expanded, hatching or hatched) on day 7 or 8 were calculated for each replicate. Treatment effects were analyzed by least-squares analysis of variance using the General Linear Models procedure of SAS (SAS for Windows, version 9.0, SAS Inst., Inc., Cary, NC). The model included the main effects of replicate and treatment. Data were analyzed two ways – as the entire data set and then separately for replicates in which blastocysts were collected at day 7 or 8 after insemination. All values reported are least-squares means ± SEM.

Logistic regression was performed using the LOGISTIC procedure of SAS to analyze data for the proportion of recipients that were pregnant at day 21 after ovulation (based on having a plasma progesterone concentration above 2.0 ng/mL), day 30 after ovulation (based on ultrasound) and day 45 after ovulation (based on rectal palpation). Calving rate and pregnancy loss were also analyzed by logistic regression. Calving rate was analyzed two ways: 1) as the proportion of recipients that gave birth to a calf, live or dead (defined as overall calving rate) and 2) as the proportion of recipients that gave birth to a calf that survived at least 24 h (defined as live calving rate). Pregnancy loss was analyzed between three time points as follows: day 21 to day 30, day 30 to day 45 and day 45 to term (except Farm 3). The models for the variables described above included the main effects of season of transfer (hot season = July, August and September and cool season = January, March, April, and November), embryo treatment, farm-season, days in milk and all two-way interactions. Additional analyses for pregnancy rate, calving rate and pregnancy loss were also conducted. One analysis included a subset of recipients at Farm 2 and Farm 3 only. These were two locations at which transfers were completed in both the cool season and the hot season. Another analysis included a subset of recipients that received embryos cultured in 5% 0_2 and were harvested on day 7. In addition, analyses were also performed separately for transfers in the cool season and hot season, respectively, with farm and embryo treatment as effects. Finally, analyses were conducted separately for control and IGF-1 treated embryos to determine effects of season. All data on pregnancies and calvings are reported as the actual percentage.

Calf birth weight and gestation length were subjected to analysis of variance using the GLM procedure of SAS. Data were analyzed for the data set of all calves and the

data set of live calves. The models included embryo treatment, sex of calf and farm-season. All values are reported as least-squares means ± SEM. The proportion of calves that were male was analyzed among all calves and all live calves using the LOGISTIC procedure of SAS. The model included season of transfer, embryo treatment, farm-season and all two-way interactions. The effect of breeding type (i.e., artificial insemination or embryo transfer) on calf birth weight and calf sex for a subset of cows at Farm 2 was also analyzed. In addition, chi-square analysis was used to determine if the sex ratio of all calves and all live calves deviated from the expected 50:50 ratio.

Results

Embryo Development

Overall, there was no effect of IGF-1 on cleavage rate at day 3 after insemination (control – 77.3 ± 0.8% vs. IGF-1 – 78.9 ± 0.8%), the proportion of oocytes that became blastocysts (control – 16.2 ± 1.3% vs. IGF-1 – 17.2 ± 1.3%), or the proportion of oocytes that became advanced blastocyst stages (expanded, hatching or hatched) (7.6 ± 0.7% vs. IGF-1 – 8.4 ± 0.7%). When only those replicates in which blastocyst development was recorded on day 8 after insemination (n = 7 replicates) were analyzed separately, there was also no effect of IGF-1 on the proportion of oocytes becoming blastocysts (control – 21.9 ± 1.6% vs. IGF-1 – 20.2 ± 1.6%) or advanced blastocysts (control – 8.9 ± 0.4% vs. IGF-1 – 8.8 ± 0.4%). However, among replicates in which blastocyst development was recorded on day 7 after insemination (n = 15 replicates), IGF-1 increased the proportion of oocytes becoming blastocysts ($P < 0.001$; control – 13.9 ± 0.4% vs. IGF-1 – 16.0 ± 0.4%) and tended to increase the proportion that became advanced blastocysts ($P < 0.07$; control – 7.1 ± 0.4% vs. IGF-1 – 8.2 ± 0.4%).

Pregnancy Rate

Using plasma progesterone concentrations greater than 2.0 ng/mL as a diagnosis of pregnancy, the proportion of cows pregnant at day 21 after ovulation was not different between recipients that received control versus IGF-1 treated embryos (Table 4-1). However, there was a tendency for an increased proportion of recipients with plasma progesterone above 2.0 ng/mL in the hot season ($P < 0.06$) compared to the cool season (Table 4-1). There was also a trend for an interaction between season and treatment ($P < 0.09$) with a higher pregnancy rate for recipients receiving IGF-1 treated embryos than recipients receiving control embryos during the hot season but not the cool season (Table 4-1).

As shown in Table 4-1, there was a season x embryo treatment interaction that affected pregnancy rate at day 30 and day 45 of gestation ($P < 0.01$). In the hot season, recipients that received IGF-1 treated embryos had higher pregnancy rates at both day 30 and day 45 than recipients receiving control embryos. In the cool season, in contrast, there was no difference between recipients receiving IGF-1 treated embryos or control embryos.

Farms 2 and 3 were the two locations where transfers were performed in both seasons. When data from these two farms only were analyzed, there was an interaction between season and IGF-1 ($P < 0.01$) for pregnancy rate at day 21 (cool season: control – 27/35 = 77.1% and IGF-1 – 2134 = 61.8%; hot season: control – 41/59 = 69.5% and IGF-1 – 51/63 = 81.0%), day 30 of gestation (cool season: control – 12/35 = 34.3% and IGF-1 – 5/34 = 14.7%; hot season: control – 15/71 = 21.1% and IGF-1 – 34/69 = 49.3%) and day 45 of gestation (cool season: control – 10/35 = 28.6% vs. IGF-1 – 6/37 = 16.2%; hot season: control – 13/71 = 18.3% vs. IGF-1 – 28/67 = 41.8%).

A third analysis considered only those embryos that were cultured in 5% oxygen and harvested at day 7 (n=15 replicates). Results are shown in Table 4-2. There was an interaction between IGF-1 and season for pregnancy rate at day 30 (P<0.06) (cool season: control – 13/40 = 32.5% and IGF-1 – 13/37 = 35.1%; hot season: control – 15/71 = 21.1% and IGF-1 – 34/69 = 49.3%) and a tendency (P < 0.09) for an interaction at day 45 (cool season: control – 11/45 = 24.4% and IGF-1 – 11/42= 26.2%; hot season: control – 13/71 = 18.3% and IGF-1 – 28/67 = 41.8%).

A fourth analysis was performed in which data were analyzed for each season separately. In this analysis, there was no effect of IGF-1 on pregnancy rate in the cool season at day 21, day 30 or day 45, but in the hot season IGF-1 treatment increased pregnancy rates at day 30 and day 45 ($P < 0.01$).

When the effect of season was analyzed for each treatment separately, there was a tendency (P < 0.08) for control embryo recipients to have lower pregnancy rates at day 30 in the hot season than the cool season (Table 4-1). There were no effects of season for pregnancy rate at day 21 or day 45. Among IGF-1 recipients, pregnancy rate at day 21, day 30 and day 45 were higher (P < 0.02) in the hot season compared to the cool season. Similar seasonal effects were apparent for the subset of cows receiving embryos collected at day 7 after insemination but differences were not significant (Table 4-2).

Calving Rate

These data were available for a subset comprising Farms 1, 2 and 4. There was an interaction ($P < 0.05$) between season and embryo treatment affecting overall calving rate and a tendency ($P < 0.11$) for an interaction affecting live calving rate (Table 4-1). At farm 2 where transfers were done in both the cool and hot seasons, there was significant interaction ($P < 0.03$) between season and embryo treatment on overall calving rate (cool

season: control – 4/13 = 30.8% vs. IGF-1 – 1/17 = 5.9%; hot season: control – 5/38 = 13.2% vs. IGF-1 – 10/30 = 33.3%) and live calving rate (cool season: control – 4/13 = 30.8% vs. IGF-1 – 1/17 = 5.9%; hot season: control – 5/38 = 13.2% vs. IGF-1 – 9/29 = 31.0%). Among recipients that received embryos that were cultured in 5% O_2 and harvested on day 7, there was a numerical interaction between season and embryo treatment for both overall calving rate and live calving rate but these differences were not statistically different (Table 4-2).

When data were analyzed from the cool season only, there was no effect of IGF-1 on overall calving rate or live calving rate (Table 4-1). However, when data from the hot season were analyzed separately, IGF-1 tended to increase both overall calving rate ($P < 0.06$) and live calving rate ($P < 0.09$). When data were analyzed separately for each treatment group, recipients that received IGF-1 treated embryos tended ($P < 0.10$) to have a higher overall calving rate in the hot season compared to the cool season, but there was no difference in live calving rate. For control embryo recipients, there was no significant effect of season on either overall or live calving rate although, numerically, calving rates were greater for the cool season.

Pregnancy Loss

Pregnancy loss was 52.8% (96/182) between day 21 and day 30 of gestation. A total of 10.8% (10/93) and 20.4% (10/49) of pregnant recipients lost their pregnancies from day 30 to day 45 and day 45 to term, respectively.

There was an interaction ($P < 0.01$) between season and embryo treatment affecting pregnancy loss from day 21 to ay 30. Pregnancy loss in the cool season was not different between recipients that received control versus IGF-1 embryos but recipients in

the hot season that received control embryos had more pregnancy loss than recipients receiving IGF-1 treated embryos (Table 4-3).

For recipients at Farm 2 and Farm 3 where transfers were done in both seasons, there was also an interaction ($P < 0.02$) between season and embryo treatment affecting pregnancy loss from day 21 to day 30 (cool season: control – 15/27 = 55.6% vs. IGF-1 – 14/19 = 73.7%; hot season: control – 29/41 = 70.7% vs. IGF-1 – 19/51 = 37.3%) and Day 21 to Day 45 (cool season: control – 16/26 = 61.5% vs. IGF-1 – 16/21 = 76.2%; hot season: control – 31/41 = 75.6% vs. IGF-1 – 23/49 = 46.9%).

Among the subset of recipients that received embryos that were cultured in 5% O_2 and harvested on day 7, pregnancy loss between day 21 and day 30 was lower ($P < 0.05$) for recipients that received IGF-1 treated embryos than for controls (Table 4-4). There was also a tendency for an interaction ($P < 0.07$) between season and IGF-1 on pregnancy loss between day 21 and day 30 (Table 4). When pregnancy loss data were analyzed from the cool season only, there was no effect of IGF-1 on pregnancy loss. However, when data from the hot season were analyzed separately, IGF-1 embryo recipients had lower pregnancy loss ($P < 0.04$) from day 21 to day 30. When data were analyzed separately among treatment groups, recipients that received control embryos had higher ($P < 0.05$) pregnancy loss between day 21 and day 30 in the hot season compared to the cool season. On the other hand, recipients that received IGF-1 treated embryos had lower pregnancy loss between day 21 and day 30 in the hot season compared to the cool season ($P < 0.06$).

Gestation Length

There were no effects of embryo treatment on gestation length whether all calves or only live calves were analyzed. Recipients that received embryos in the hot season

had shorter ($P < 0.04$) gestation lengths than recipients that received embryos in the cool season (all calves: cool season – 278.8 ± 1.1 days vs. hot season - 274.3 ± 1.4 days; live calves: cool season – 278.5 ± 1.1 days vs. hot season – 274.4 ± 1.4 days).

Calf Sex Ratio and Birth Weight

The calf sex ratio was different ($P < 0.002$) than the expected 50:50 ratio. In particular, there was a preponderance of male calves among all calves born (31/40 = 77.5%) as well as live calves only (28/37 = 75.7%). There were no effects of embryo treatment, season of transfer, farm-season or gestation length on calf sex ratio (Table 4-3). The proportion of male calves born following artificial insemination at Farm 2 was 50% for all calves (27/54) as well as all live calves (26/52). This was significantly lower ($P < 0.04$) than the proportion of male calves born following embryo transfer at Farm 2 (all calves: 16/20 = 80% and live calves: 15/19 = 79.0%).

Calf birth weight was recorded for 20 calves at Farm 2. There were no effects of embryo treatment, season of transfer, or calf sex on calf birth weight (Table 4-3). Of the 20 calves, one was born dead. This calf was from the IGF-1 treatment group and weighed 68.2 kg at birth. For the 19 calves born alive, there was also no effect of embryo treatment, season of transfer, or calf sex on calf birth weight (Table 4-3). Calves born following artificial insemination at Farm 2 had lower ($P < 0.001$) birth weights than for calves born following embryo transfer. This was true for all calves (artificial insemination – 41.1 ± 0.8 vs. embryo transfer – 48.2 ± 1.3 kg) as well as all live calves (artificial insemination – 41.2 ± 0.8 vs. embryo transfer – 47.1 ± 1.3 kg).

Discussion

The objective of the present experiment was to determine whether culturing embryos in the presence of IGF-1 would increase pregnancy and calving rates following

the transfer of in-vitro produced bovine embryos to lactating dairy cows. Results indicate that pregnancy and calving rates can be increased by IGF-1 in the hot season but not the cool season. While heat stress tended to reduce post-transfer survival of control embryos, treatment of embryos with IGF-1 blocked this effect and, in fact, caused an increase in pregnancy rate greater than the reduction caused by heat stress. The calves born as a result of IGF-1 treatment were similar to those derived from control embryos. Thus, IGF-1 treatment can improve the efficacy of in-vitro embryo transfer during summer without additional alterations in gestation length or calf birth weight. Results also point out however, some limitations to the transfer of in-vitro produced embryo, including high fetal loss, increased calf birth weight, and skewed sex ratio.

While treatment of embryos with IGF-1 improved embryo survival following transfer in the hot season, there was no effect of IGF-1 treatment on pregnancy and calving rates in the cool season. The interaction between embryo treatment and season of transfer on pregnancy rates also occurred among a subset of recipients at Farms 2 and 3 where transfers were done in both seasons, as well as among recipients that received embryos that were cultured in 5% O_2 and harvested on Day 7. In addition, when data from the cool season were analyzed there was no effect of IGF-1 on pregnancy and calving rates. In contrast, when data from the hot season were analyzed IGF-1 embryo treatment increased pregnancy and calving rates. The finding that IGF-1 increased pregnancy rate in the hot season agrees with a previous report in which treatment of embryos with IGF-1 increased pregnancy and calving rates in heat-stressed, lactating dairy cows (Block et al., 2003).

The mechanism by which IGF-1 improves post-transfer embryo survival during heat stress is not known. However, IGF-1 is a survival factor for the preimplantation embryo and can reduce deleterious effects of heat shock on development to the blastocyst stage and apoptosis (Jousan and Hansen, 2004, 2006). Although embryos have acquired substantial resistance to elevated temperature by the blastocyst stage of development (Ealy et al., 1993; Edwards and Hansen, 1997, results from the current study and others (Vasconcelos et al., 2006; Galvao et al., 2006) indicate that there is a reduction in post-transfer survival of embryos during heat stress. Such an effect could represent actions on the embryo or mother (for example, reduced blood concentrations of progesterone, Wolfenson et al., 2000). One possibility is that the increased survival for IGF-1 treated embryos represents an improved capacity of the embryo to withstand exposure to maternal hyperthermia following transfer.

It is also possible that IGF-1 alters developmental processes in a way that results in blastocysts with increased capacity for survival when maternal function is compromised (as may be the case during heat stress). An increase in embryo development to the blastocyst stage following addition of IGF-1 to bovine embryo culture medium has been reported many times (Palma et al., 1997; Prelle et al., 2001; Byrne et al., 2002b; Moreira et al., 2002b; Block et al., 2003; Sirisathien et al., 2003b). In the present study, IGF-1 treatment increased blastocyst development on day 7 after insemination but had no effect on day 8. Although statistically significant, the increase in blastocyst development on Day 7 was only 2.1%. This is similar to the increase in embryo development for IGF-1 treated embryos observed in a previous report from our laboratory (Block et al., 2003), but smaller than previous reports with IGF-1 (Byrne et al.,

2002b; Makeravich and Markkula, 2002; Moreira et al., 2002b; Sirisathien et al., 2003b). Differences in the effect of IGF-1 on embryo development may be partly explained by differences in culture systems because there are reports that effects of IGF-1 on embryonic development depend upon culture conditions (Herrler et al., 1992; Palma et al., 1997).

The effects of IGF-1 to increase pregnancy rate in the summer involve more than simply reversing the deleterious effects of season on embryonic survival. This is so because pregnancy and calving rates for IGF-1 embryo recipients in the hot season were higher than the pregnancy and calving rates of the control embryo recipients in the cool season. It is not clear at the present time why there would be a synergistic effect between IGF-1 and heat stress on embryo survival. Perhaps positive effects of IGF-1 can be offset by other actions of IGF-1 that reduce embryonic survival and the predominating effect (positive, negative, or no effect) depends upon characteristics of the oocyte used to produce embryos or the recipient. Indirect evidence for this idea comes from studies with the IGF-1 secretagogue, bovine somatotropin. Administration of somatotropin can increase the proportion of cows pregnant following timed artificial insemination if cows are lactating (Moreira et al., 2000; Moreira et al., 2001; Santos et al., 2004). In contrast, somatotropin administration decreased the proportion of non-lactating cows pregnant following timed artificial insemination (Bilby et al., 2004).

One possibility is that IGF-1 treated embryos are able to overcome alterations in uterine function caused by heat stress. For example, the secretion of prostaglandin $F_{2\alpha}$ from the endometrium of pregnant cows is increased by heat shock (Putney et al., 1988). Since IGF-1 treated embryos can be more advanced in development (Moreira et al.,

2002b; Block et al., 2003) and have increased cell numbers (Byrne et al., 2002b; Moreira et al., 2002b; Sirisathien et al., 2003b) they may be able to block this increase in PGF secretion by producing more IFN-τ. Conversely, during cool periods when PGF secretion is less likely to be altered, this effect of IGF-1 may not be beneficial.

Overall pregnancy loss between day 21 and term in the present study was 70.2% (80/114). A total of 50.2% (96/182) of pregnancies were lost between day 21 and day 30 of gestation; this period is thus a major source of pregnancy loss. It is likely that the day 21 pregnancy rate is an overestimate and therefore should be interpreted carefully. Other factors such as recipient asynchrony, extended estrous cycles (> 21 days), luteal cysts and subclinical uterine infections could have contributed to elevated plasma progesterone. It is also important to note, however, that similar pregnancy losses between day 21-22 and day 42-52 have been reported in lactating dairy cows following artificial insemination and embryo transfer (Ambrose et al., 1999; Drost et al., 1999; Chebel et al., 2004).

Interestingly, day 21 to day 30 of gestation was also the time during which IGF-1 had a major effect on embryo survival. The beneficial effect of IGF-1 on embryo survival during this time period was only evident during the hot season. While there was no difference in pregnancy loss between IGF-1 and control embryos from day 21 to day 30 in the cool season (57.1% vs. 50.0%, respectively), there was significantly less pregnancy loss from day 21 to day 30 for IGF-1embryos compared to controls during the hot season (37.3% vs. 70.7%, respectively). This result suggests that IGF-1 treatment from day 1-7 after insemination is affecting events after the time of maternal recognition of pregnancy and during the peri-attachment period of gestation. These events could include overall growth of the embryo or the program of gene expression. One possibility

is that IGF-1 treatment increases conceptus size but this effect is only beneficial for embryo survival under stressful conditions such as hyperthermia. Such a dichotomy has been observed for the effect of somatotropin on conceptus length and pregnancy rates in dairy cattle. Although somatotropin treatment increases conceptus length at day 17 in both lactating and non-lactating dairy cows, only lactating dairy cows have improved pregnancy rates following somatotropin treatment (Bilby et al., 2004, 2006). Another possible explanation involves the formation of the embryonic disc. While only 35-72.6% of in vitro produced embryos recovered at day 14-16 have a detectable embryonic disc (Rexroad and Powell, 1999; Fischer-Brown et al., 2005), the addition of IGF-1 to embryo culture has been reported to increase the number of cells in the inner cell mass (Sirisathien et al., 2003). Thus IGF-1 treatment may result in a more viable embryonic disc which is more capable of withstanding heat stress.

Embryonic loss between day 30 and day 45 was 10.8% and this value is within the range reported for lactating dairy cows following artificial insemination (Chebel et al., 2004; Santos et al., 2004; Sartori et al., 2006;Vasconcelos et al., 2006) or embryo transfer with superovulated embryos (Sartori et al., 2006; Vasoncelos et al., 2006) during similar time periods. Fetal loss (from day 45 to calving) in the present study was 20.4%. In a previous report from our laboratory in which in-vitro produced embryos were transferred to lactating dairy cows, pregnancy loss from day 53 of gestation to calving was 24.0% (Block et al., 2003). These values are high compared to values ranging from 7.6-13.1% for fetal loss between day 50-60 of gestation and calving for pregnancies established with in vitro produced embryos (Hasler et al., 2000; Heyman et al., 2002) and values of 10.0% for fetal loss rate between day 40-50 of gestation and term for lactating

cows in Florida bred by artificial insemination (Jousan et al., 2005). It is also possible that the oocyte or culture system used to produce embryos resulted in a large proportion of conceptuses incapable of completing fetal development. Another possible contributing factor is lactational status because lactating dairy cows were used as recipients here compared with the heifer recipients used elsewhere (Hasler et al., 2000; Heyman et al., 2002(. Fetal losses in females impregnanted by artificial insemination are higher in lactating cows than heifers (Jousan et al., 2005). In another study from our laboratory, pregnancy losses between Day 67 of gestation and term were 6.7% when single in vitro-produced embryos were transferred into heifers or crossbred dairy cows producing low amounts of milk (Franco et al., 2006a).

The sex ratio of calves born in the present study was significantly different from the sex ratio of calves born following artificial insemination as well as the expected 50:50 ratio with 31/40 (77.5%) calves being male. Several previous studies have reported a skewed sex ratio in favor of males following the transfer of in-vitro produced embryos with a range of 55.4 to 82.0% (Massip et al., 1996; van Wagtendonk et al., 1998; Hasler et al., 2000). The sex ratio of 77.5% in this study is higher than that reported in a previous study from our laboratory in which the sex ratio was 64.3% males (Block et al., 2003). The increase in the proportion of male calves in this study is likely due to the fact that most of the embryos in the present study were selected on day 7 following insemination compared to day 8 in the previous report. Male embryos develop to the blastocyst stage in vitro faster than female embryos (Avery et al., 1991; Xu et al.,1992). In addition, the high proportion of male calves is most likely due in large part to a skewed sex ratio at the time of embryo selection. The sex ratio of in vitro produced embryos at

or beyond the morula stage in our laboratory was 69.5% males (Block et al., 2003). While an increase in the maximum air temperature around the time of conception has been associated with an increase in the proportion of male calves (Roche et al., 2006), there was no effect of season on calf sex ratio in the present study.

The mean birth weight of embryo transfer calves was 6-7 kg higher than calves born following artificial insemination. This result is consistent with previous reports in which birth weights of calves produced following in-vitro embryo production were higher than for calves produced following artificial insemination (van Wagtendonk et al., 1998; 2000). Caution must be used in interpreting the observed difference because sires differed between embryo transfer calves and artificial insemination calves. Although addition of IGF-1 to bovine embryo culture has been reported to increase blastocyst cell number (Byrne et al., 2002b; Moreira et al., 2002b; Sirisathien et al., 2003b), IGF-1 had no effect on calf birth weight. This result agrees with a previous report from our laboratory in which IGF-1 treatment improved pregnancy rates but did not alter calf birth weight (Block et al., 2003).

There are important practical implications of the present findings. Embryo transfer has been proposed as a tool for increasing pregnancy rate in the summer because the embryo becomes more resistant to elevated temperature as it advances in development (Ealy et al., 1993; Edwards and Hansen, 1997). Indeed, use of embryo transfer has been shown to improve pregnancy rates during heat stress in Florida (Putney et al., 1989; Ambrose et al., 1999; Drost et al., 1999; Al-Katanani et al., 2002 and Brazil (Rodrigues et al., 2004). While embryos can be produced following superovulation, in-vitro embryo production can be a more practical alternative for the large scale production

of embryos (Bousquet et al., 1999). The improvement in pregnancy rates caused by culture with IGF-1 resulted in a pregnancy rate at day 45 for embryo recipients in the hot season of 41.8% (28/67). This pregnancy rate is much higher than the pregnancy rates of 14-19% in two previous studies evaluating the effectiveness of in vitro embryo transfer in the summer (Ambrose et al., 1999; Al-Katanani et al., 2002). This result suggests that addition of IGF-1 to embryo culture can improve the effectiveness of in-vitro embryo transfer in the summer when compared to artificial insemination and result in pregnancy rates comparable to those achieved using artificial insemination in cool weather.

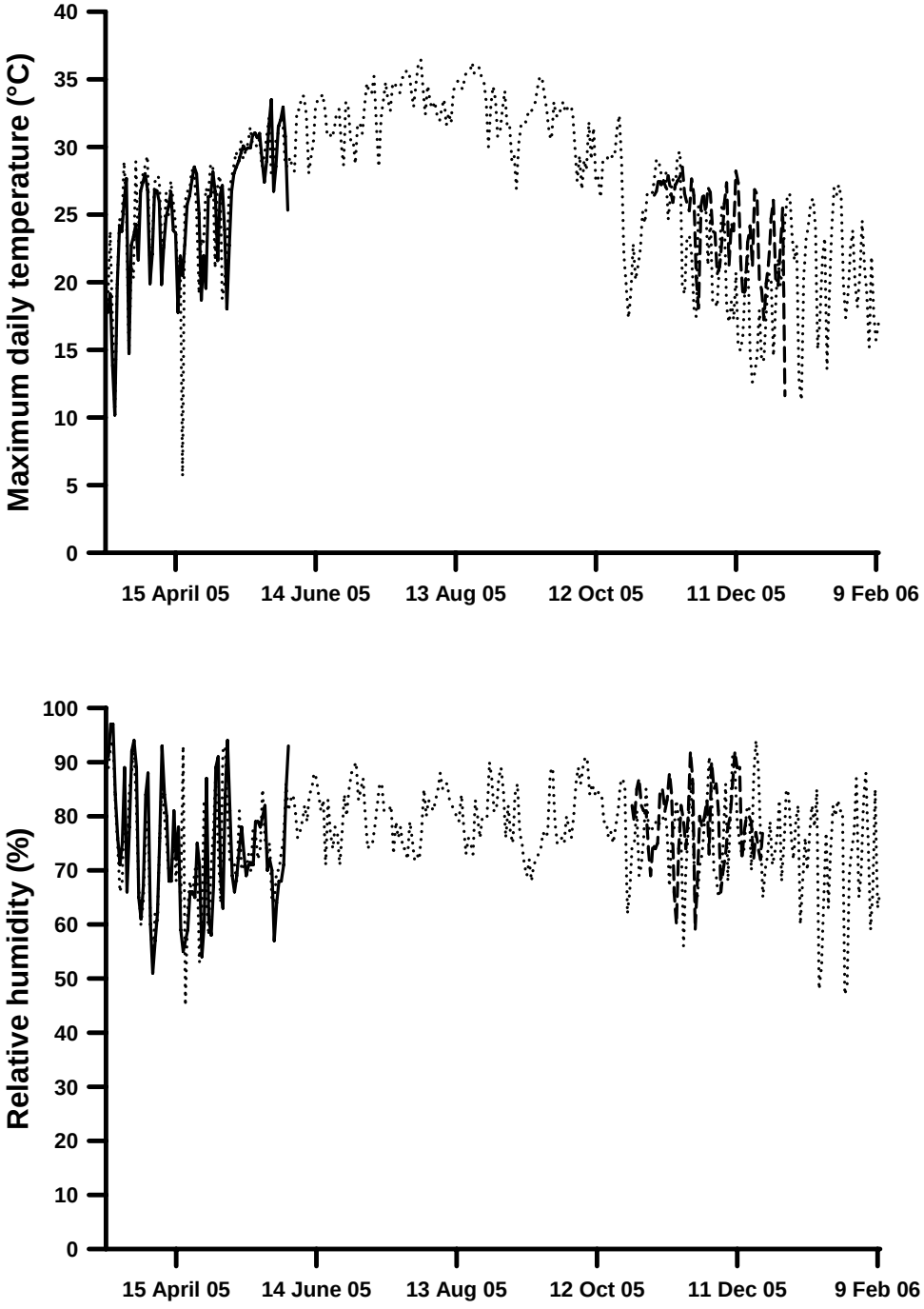

Figure 4-1. Daily maximal dry bulb temperatures (top panel) and daily relative humidity (bottom panel) for Farm 1 (solid line), Farms 2 and 3 (dotted line) and Farm 4 (dashed line) from March 15, 2005 to February 9th, 2006.

Table 4-1. Effect of season and IGF-1 on pregnancy rate at Day 21 (based on elevated plasma progesterone concentrations), Day 30 (based on ultrasound) and Day 45 of gestation (based on rectal palpation) and calving rate for all recipients.

	Pregnancy rate, number pregnant/total (%)[a]			Calving rate, number calving/total (%)[b]	
	Day 21[c]	Day 30[df]	Day 45[ef]	All calves[g]	Live calves[h]
Cool season					
Control	51/76 = 67.1%	27/79 = 34.2%	24/87 = 27.6%	14/62 = 22.6%	12/60 = 20.0%
IGF-1	46/74 = 62.2%	21/77 = 27.3%	19/83 = 22.9%	11/62 = 17.7%	11/62 = 17.7%
Hot season					
Control	41/59 = 69.5%	15/71 = 21.1%	13/71 = 18.3%	5/38 = 13.2%	5/38 = 13.2%
IGF-1	51/63 = 81.0%	34/69 = 49.3%	28/67 = 41.8%	10/30 = 33.3%	9/29 = 31.0%

[a] Differences in the number of recipients at each time point is due to some recipients not being diagnosed for pregnancy at all time points. [b] Data does not include Farm 3. [c] Season x treatment $P < 0.09$. [d] Treatment $P < 0.06$. [e] Treatment $P < 0.07$. [f] Season x treatment $P < 0.01$. [g] Season x treatment $P < 0.05$. [h] Season x treatment $P < 0.11$.

Table 4-2. Effect of season and IGF-1 on pregnancy rate at Day 21 (based on elevated plasma progesterone concentrations), Day 30 (based on ultrasound) and Day 45 of gestation (based on rectal palpation) and calving rate among recipients that received embryos that were cultured in 5% O_2 and harvested on Day 7.

	Pregnancy rate, number pregnant/total (%)[a]			Calving rate, number calving/total (%)[b]	
	Day 21	Day 30[ce]	Day 45[df]	All calves	Live calves
Cool season					
Control	27/42 = 64.3%	13/40 = 32.5%	11/45 = 24.4%	5/22 = 22.7%	4/21 = 19.1%
IGF-1	27/39 = 69.2%	13/37 = 35.1%	11/42 = 26.2%	5/22 = 22.7%	5/22 = 22.7%
Hot season					
Control	41/59 = 69.5%	15/71 = 21.1%	13/71 = 18.3%	5/38 = 13.2%	5/38 = 13.2%
IGF-1	51/63 = 81.0%	34/69 = 49.3%	28/67 = 41.8%	10/30 = 33.3%	9/29 = 31.0%

[a] Differences in the number of recipients at each time point is due to some recipients not being diagnosed for pregnancy at all time points. [b] Data does not include Farm 3. [c] Treatment $P < 0.05$. [e] Season x treatment $P < 0.06$. [f] Season x treatment $P < 0.09$.

Table 4-3. Effect of season and IGF-1 on pregnancy loss among all recipients.

	Pregnancy loss, number of losses/total pregnancies (%)[a]		
	Day 21 to Day 30[cd]	Day 30 to Day 45	Day 45 to term[b]
Cool season			
Control	24/48 = 50.0%	3/26 = 11.5%	2/15 = 13.3%
IGF-1	24/42 = 57.1%	3/22 = 13.6%	2/13 = 15.4%
Hot season			
Control	29/41 = 70.7%	1/14 = 7.1%	4/9 = 44.4%
IGF-1	19/51 = 37.3%	3/31 = 9.7%	2/12 = 16.7%

[a] Differences in the number of pregnancies at each time point is due to some recipients not being diagnosed for pregnancy at all time points as well as not having calving data for Farm 3. [b] Data does not include Farm 3. [c] Treatment $P < 0.07$. [d] Season x treatment $P < 0.01$.

Table 4-4. Effect of season and IGF-1 on pregnancy loss among recipients that received embryos that were cultured in 5% O_2 and harvested on Day 7.

	Pregnancy loss, number of losses/total pregnancies (%)[a]		
	Day 21 to Day 30[cd]	Day 30 to Day 45	Day 45 to term[b]
Cool season			
Control	13/25 = 52.0%	2/12 = 16.7%	0/5 = 0%
IGF-1	12/24 = 50.0%	3/14 = 21.4%	1/6 = 16.7%
Hot season			
Control	29/41 = 70.7%	1/14 = 7.1%	4/9 = 44.4%
IGF-1	19/51 = 37.3%	3/31 = 9.7%	2/12 = 16.7%

[a] Differences in the number of pregnancies at each time point is due to some recipients not being diagnosed for pregnancy at all time points as well as not having calving data for Farm 3. [b]Data does not include Farm 3. [c] Treatment $P < 0.05$. [d] Season x treatment $P < 0.07$.

Table 4-5. Effect of IGF-1 on calf birth weight and sex ratio

	Birth weight (kg)	n	Male calves (%)
All calves			
Control	46.6 ± 3.0	9	15/19 = 79.0%
IGF-1	48.2 ± 2.9	11	16/21 = 76.2%
Live calves			
Control	47.4 ± 2.2	9	13/17 = 76.5%
IGF-1	46.8 ± 2.1	10	15/20 = 76.0%

CHAPTER 5
GENERAL DISCUSSION

Improvements in the techniques for in vitro embryo production in cattle have led to a 6 fold increase in the number of in vitro produced bovine embryos transferred worldwide over the past 5 years (Thibier, 2001, 2006). There is also great potential for future growth of the commercial in vitro embryo transfer industry in beef and dairy production systems. Incorporation of in vitro embryo production with emerging technologies, such as marker-assisted selection and sexed semen, can help to optimize genetic selection for specific traits important for meat and milk production. Moreover, the efficiency of in vitro embryo production compared to superovulation (Bousquet et al., 1999) could make it a useful tool for overcoming problems of fertility in dairy cattle, as well as, enhancing breeding schemes that involve crossbreeding and twinning.

While there is great potential for in vitro embryo production, embryos produced following superovulation still represent the bulk of embryos transferred worldwide (Thibier, 2006). One reason for the limited use of in vitro embryo technologies in cattle production systems is the fact that embryos produced in vitro have altered ultrastructural (Crosier et al., 2001; Fair at al., 2001) and physiological (Khurana and Niemann, 2000; Lonergan et al., 2006) features compared to embryos produced in vivo. As a result, post-culture viability can be compromised. In particular, bovine embryos produced in vitro are more sensitive to cryopreservation (Pollard and Leibo, 1993; Enright et al., 2000, Rizos et al., 2002) have reduced embryo and fetal survival following transfer (Farin and Farin, 1995; Hasler et al., 1995), and result in an increased number of fetuses and calves with abnormalities (Farin and Farin, 1995; van Wagtendonk de-Leeuw et al., 1998, 2000).

Studies using the sheep oviduct as a model for in vivo embryo development have highlighted the sub-optimal nature of embryo culture and the consequences of such an environment for post-culture viability (Lonergan et al., 2006). Specifically, placement of bovine embryos produced by in vitro fertilization in the sheep oviduct improves cryotolerance (Enright et al., 2000; Rizos et al., 2002), increases pregnancy rates and reduces calf birth weights (Lazzari et al., 2002) compared to embryos produced totally in vitro. These results suggest that a potential strategy for improving in vitro produced embryos is to modify culture conditions to more closely mimic the microenvironment found in vivo.

One approach to modify embryo culture systems is to add growth factor or cytokine molecules. These molecules are involved in preimplantation embryo development in vivo (Kane et al., 1997; Diaz-Cueto and Gerton, 1998; Hardy and Spanos, 2002), can have beneficial effects on embryo development in vitro (Kane et al., 1997; Diaz-Cueto and Gerton, 1998; Hardy and Spanos, 2002) and in a few cases have been reported improve embryo survival after transfer (Block et al., 2003; Roudebush et al., 2004; Sjoblom et al., 2005). In cattle, the addition of IGF-1 to embryo culture has been reported to increase pregnancy and calving rates in heat-stressed, lactating dairy cows (Block et al., 2003). The overall goal of this dissertation was to develop a better understanding of how addition of IGF-1 to embryo culture improves embryo survival post-transfer. In particular, this dissertation focused on 3 major questions as follows:

1) *What actions does IGF-1 exert during embryo development in vitro that allows for increased embryo survival after transfer?*

In Chapter 2, the effect of IGF-1 during embryo culture on the mRNA abundance for 14 developmentally important transcripts, as well as, cell number, cell allocation and apoptosis were analyzed. The results indicate that addition of IGF-1 to embryo culture does alter the mRNA

abundance of certain transcripts in bovine expanded blastocysts. In particular, embryos cultured with IGF-1 have an increased abundance of Na/K, Dc II, Bax, and IGFBP3 and a decreased abundance of Hsp70 and IGF-1R. In contrast, IGF-1 treatment did not affect blastocyst cell number, cell allocation or apoptosis.

2) *What actions does IGF-1 have on post-transfer embryo development that allow for improved embryo survival?*

In Chapter 3, two experiments were conducted to test whether IGF-1 treatment could enhance embryo survival and development at day 14 of gestation. When embryos were transferred individually to recipients, IGF-1 tended to increase embryo survival, but this effect was not observed when embryos were transferred in groups. Regardless of the number of embryos transferred, IGF-1 treatment did not affect embryo length, stage, embryonic disc formation or IFN- τ secretion.

3) *Are the effects of IGF-1 on embryo survival a general effect of IGF-1 or one specific to heat stress?*

In Chapter 4, a field trial was conducted in which control and IGF-1 treated embryos were transferred in both the hot and cool seasons. The results indicate that IGF-1 treatment can increase pregnancy and calving rates in heat stressed, lactating dairy cows, however, there was no effect of IGF-1 on embryo survival when recipients were not heat stressed. The main action of IGF-1 on embryo survival in this experiment occurred between day 21 and day 28 of gestation. Specifically, recipients that received IGF-1 treated embryos had less pregnancy loss during this period than for recipients that received control embryos.

Taken together, the results of this dissertation indicate that IGF-1 can act at multiple levels and time points to affect embryo survival post-transfer. A model that illustrates the actions of

IGF-1 on embryo development and survival, as well as, the potential mechanisms involved is depicted in Figure 5-1.

Treatment of embryos with IGF-1 altered the relative abundance of several mRNA transcripts in Chapter 2. This action of IGF-1 could be important for embryo survival at day 14 as well as at day 21-28 of gestation. Embryos produced in vitro have altered gene expression patterns compared to embryos produced in vivo (Wrenzycki et al., 1996; Wrenzycki et al., 1998; Lazzari et al., 2002; Rizos et al., 2002 Sagirkaya et al., 2006). In general, embryos produced in vitro have an increased abundance of Hsp70 (Lazzari et al., 2002; Sagirkaya et al., 2006) and a reduced abundance of the tight junction protein connexin-43 (Wrenzycki et al., 1996; Wrenzycki et al., 1998; Rizos et al., 2002). The results of Chapter 2 indicate that embryos cultured with IGF-1 have an increased abundance of Na/K and Dc II and a reduced abundance of Hsp70 (Figure 5-1A). Desmocollin II is involved in the formation of desmosomes and these play a critical role in stabilizing the TE during blastocyst formation and expansion (Fleming et al., 1991; Collins et al., 1995). In addition, Na/K regulates the accumulation of fluid in the blastoceole (Watson and Barcroft, 2001) as well as the formation of tight junctions during blastocyst expansion (Violette et al., 2006). The reduced abundance of Hsp70 transcripts in Chapter 2 is consistent with the idea that IGF-1 can reduce cellular stress (Jousan and Hansen, 2004, 2006; Kurzawa et al., 2002). Taken together, such effects could contribute to higher survival (Figure 5-1B). In contrast to the effects of IGF-1 on mRNA abundance, IGF-1 treatment did not affect blastocyst cell number, cell allocation or apoptosis. These results suggest that such characteristics may not be important for the enhanced survival of IGF-1 treated embryos (Figure 5-1C).

The results of Chapter 3 indicate that IGF-1 treatment can increase embryo survival at day 14 of gestation. This result is in contradiction to the results of Chapter 4 in which IGF-1 increased pregnancy rates but only during the hot season. The results of this dissertation are not able to clarify this discrepancy. It cannot be ruled out, that in the absence of heat stress, more IGF-1 embryos are lost after day 14. Although not statistically significant, a greater percentage of control embryos were at the tubular and filamentous stage of development (90.1% vs. 75% for control and IGF-1, respectively) while more IGF-1 treated embryos were at the spherical and ovoid stage (8.3% vs. 25.1% for control and IGF-1, respectively). Alternatively, the effect of IGF-1 treatment could depend upon the physiological status of the recipient in ways that extend beyond heat stress and recipients used in Chapter 3 were responsive to treatment. In any case, IGF-1 treatment did not affect embryo length, stage or IFN-τ secretion (Figure 5-1D). These results suggest that IGF-1 does not increase pregnancy rates by enhancing embryonic capacity to inhibit luteolysis.

In Chapter 4, IGF-1 treatment increased pregnancy and calving rates in the summer, but not the winter (Figure 5-1E). The mechanism by which IGF-1 improved post-transfer embryo survival during heat stress while not having an effect during the cool season is not known. While early embryos become more resistant as development progresses (Ealy et al., 1993; Edwards and Hansen, 1997), there does remain some detrimental effects of heat stress on pregnancy rates in embryo transfer recipients (Galvao et al., 2006; Vasconcelos et al., 2006; Chapter 4). Treatment of bovine embryos with IGF-1 reduces the deleterious effects of heat shock on bovine morula with respect to development to the blastocyst stage and apoptosis (Jousan and Hansen, 2004, 2007). Thus, supplementation of culture with IGF-1 may alter developmental processes in a way that results in embryos with an increased capacity for survival during heat stress. Another

possibility is that IGF-1 can have either positive or negative effects on embryo survival and the predominating effect (positive, negative, or no effect) is dependent on other physiological stimuli, such as heat stress in this case. Indirect evidence for this idea comes from studies with bST. Administration of bST can increase conceptus length and pregnancy rates following timed artificial insemination if cows are lactating (Bilby et al., 2006). For non-lactating cows, in contrast, bST increases conceptus length but reduces pregnancy rates following timed artificial insemination (Bilby et al., 2004)

One of the main actions of IGF-1 in Chapter 4 was to promote embryo survival between day 21 and day 28 of gestation in the summer. This finding suggests that the treatment of embryos with IGF-1 from Day 1-7 after fertilization can affect events after the time of maternal recognition of pregnancy and during the peri-attachment period of gestation. The fact that IGF-1 altered the mRNA abundance of some gene transcripts in bovine expanded blastocysts in Chapter 2 may help explain such an effect. It is well recognized that embryo culture conditions can alter mRNA expression and that these alterations have implications for fetal and neonatal development (Wrenzycki et al., 2005; Farin et al., 2006). Production of bovine embryos in vitro can alter the expression of both imprinted (IGF-II; Blondin et al., 2000) and non-imprinted genes (myostatin - Crosier et al., 2002; VEGF - Miles et al., 2005) as late as day 70 of gestation. Altered post-culture development is also associated with epigenetic modifications during early embryo development, such as altered methylation patterns (Fleming et al., 2004). A recent study indicates that certain bovine embryo culture media can alter the abundance of DNA methyltransferase 3a (Sagirkaya et al., 2006), an enzyme involved in re-estabishing methylation patterns during early embryo development (Reik et al., 2001). One possibility is that the presence of IGF-1 during early embryo development affects DNA methylation patterns to allow

for expression of genes important for embryo survival after the period of maternal recognition of pregnancy (Figure 5-1F).

The actions of IGF-1 to promote embryo survival during heat stress have implications for the use of in vitro embryo transfer in the summer. Use of embryo transfer has been shown to improve pregnancy rates during heat stress in Florida (Putney et al., 1989; Ambrose et al., 1999; Drost et al., 1999; Al-Katanani et al., 2002) and Brazil (Rodrigues et al., 2004). While embryos can be produced following superovulation, in-vitro embryo production can be a more practical alternative for the large scale production of embryos (Bousquet et al., 1999). Transfer of embryos cultured with IGF-1 resulted in a pregnancy rate at Day 45 of 41.8% (Chapter 4). This result suggests that addition of IGF-1 to embryo culture can improve the effectiveness of in-vitro embryo transfer in the summer so that pregnancy rates can be achieved that are comparable to those using artificial insemination in cool weather.

In addition, the ability to increase pregnancy rates by culturing embryos in the presence of IGF-1 also has implications for manipulating IGF-1 concentrations in the whole animal to increase pregnancy rates during heat stress. Administration of bST or feeding propylene glycol, which can increase circulating concentrations of IGF-1 (Formiqoni et al., 1996; Bilby et al., 2006) and improve pregnancy rates in cattle that are not heat stressed (Moreira et al., 2000; Hidalgo et al., 2004), may be useful for enhancing embryo survival in the summer.

The results of this dissertation along with a previous report (Block et al., 2003) indicate that addition of growth factors to bovine embryo culture can be used as a strategy to increase post-transfer embryo survival. The fact that IGF-1 only seems to improve embryo survival in the summer suggests that other growth factors or cytokine molecules should be evaluated for their effects on embryo survival. A potential candidate is granulocyte-macrophage stimulating

hormone which can enhance bovine embryo development in vitro (de Moraes et al., 1997) and has been reported to increase birth rate in mice (Sjoblom et al., 2005).

Future experiments evaluating the effects of IGF-1 on embryo survival should focus, in more depth, on the molecular and cellular effects of IGF-1 supplementation during embryo culture. In particular, the evaluation of gene expression and methylation patterns at different time points, such as at day 7 and d 25, may provide more insight in to the actions of IGF-1 to promote embryo survival between day 21 and day 28 of gestation. In addition, the use of more advanced techniques, such as microarray or SELDI-TOF to evaluate IGF-1 actions on a larger scale could be beneficial in identifying markers of embryo survival. Such markers could potentially be useful in developing assays to select embryos prior to transfer.

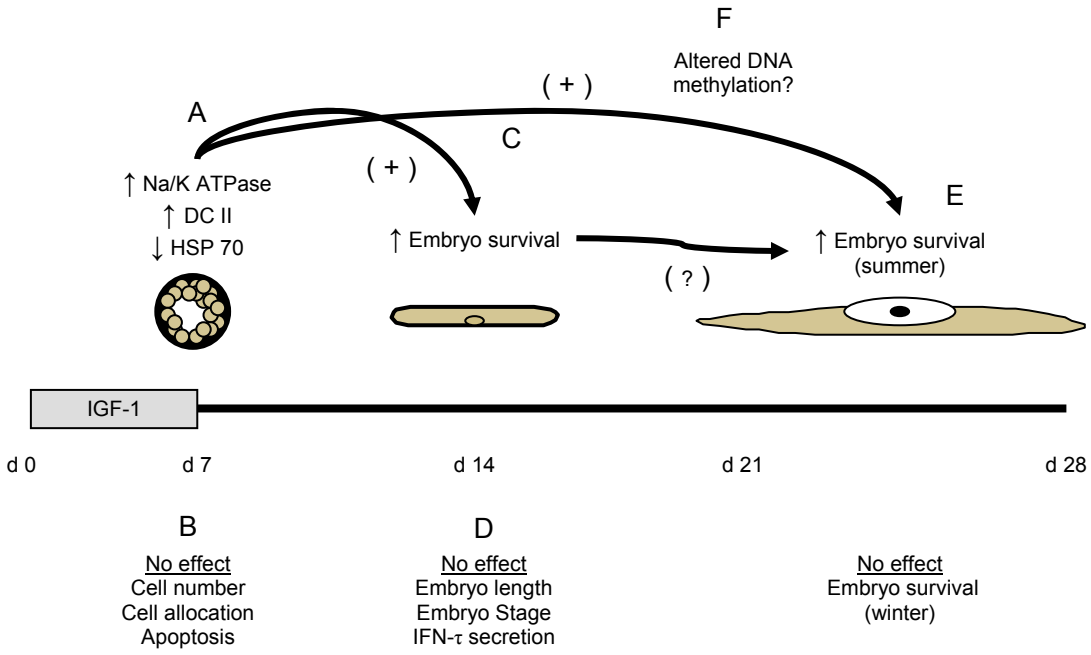

Figure 5-1. Summary of the effects of IGF-1 on embryo development and post-transfer survival. See text for details.

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BIOGRAPHICAL SKETCH

Jeremy Block was born on February 16th, 1977, to Chris and Janet Block. The elder of two children, Jeremy is a native of Wellington, Missouri, where he graduated from Wellington-Napoleon High School with honors in 1995. Following graduation from high school, he attended the University of Missouri in Columbia, Missouri, and earned a Bachelor of Science degree in animal sciences in the fall of 1998. In August of 2000, Jeremy moved to Florida and began work on a Master of Science degree in animal sciences at the University of Florida in Gainesville. His master's research focused on in vitro embryo production in cattle under the supervision of Dr. Peter J. Hansen. After completing his master's degree in the summer of 2003, Jeremy was awarded a University of Florida Graduate Alumni Fellowship and continued work with Dr. Hansen on a Doctor of Philosophy degree in the Animal Molecular and Cell Biology Graduate Program. After completing the requirements for his doctoral degree, Jeremy will begin a career in the commercial embryo transfer industry.